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(71) Applicant (*for all designated States except US*): AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): BOOTS, Anna, Maria, Helena [NL/NL]; Verlengde Torenstraat 10, NL-5366 AV Megen (NL). VERHEIJDEN, Gijsbertus, Franciscus, Maria [NL/NL]; Heischouw 7, NL-5345 XT Oss (NL).

(74) Agent: HERMANS, F., G., M.; Postbus 20, NL-5340 BH Oss (NL).

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(57) Abstract

The present invention relates to novel peptides derived from the autoantigen HC gp-39, said peptides comprising at least one of the amino acid sequences FGRSFTLAS (SEQ ID No.1), FTLASSETG (SEQ ID No. 2), YDDQESVKS (SEQ ID No.3) and FSKIASNTQ (SEQ ID No.4). The peptides resemble MHC Class II restricted T-cell epitopes present on the autoantigen HC gp-39 in articular cartilage. HC gp-39 and said peptides can be used in antigen-specific treatment of articular cartilage destruction in autoimmune diseases to induce tolerance of the immune system. The autoantigen HC gp-39 and said peptides are also suitable to induce arthritis in non-human animals, preferably mice. The invention furthermore relates to pharmaceutical compositions comprising said autoantigen and/or said peptides, a diagnostic method for the detection of autoreactive T cells in a test sample and test kits to be used in said method.

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NOVEL PEPTIDES DERIVED FROM AUTOANTIGEN FOR USE IN IMMUNOTHERAPY OF AUTOIMMUNE DISEASES

The invention relates to a novel autoantigen and peptides derived thereof, their use in treatment of chronic destruction of articular cartilage in autoimmune diseases, pharmaceutical compositions comprising said peptides, a diagnostic method for the detection of autoreactive T cells in a test sample and test kits to be used in said method.

The immune system is established on a principle of discrimination between foreign antigens (non-self antigens) and autoantigens (self antigens, derived from the individuals own body) achieved by a build in tolerance against the autoantigens.

The immune system protects individuals against foreign antigens and responds to exposure to a foreign antigen by activating specific cells such as T- and B lymphocytes and producing soluble factors like interleukins, antibodies and complement factors. The antigen to which the immune system responds is degraded by the antigen presenting cells (APCs) and a fragment of the antigen is expressed on the cell surface associated with a major histocompatibility complex (MHC) class II glycoprotein. The MHC-glycoprotein-antigen-fragment complex is presented to a T cell which by virtue of its T cell receptor recognizes the antigen fragment conjointly with the MHC class II protein to which it is bound. The T cell becomes activated, i.e. proliferates and/or produces interleukins, resulting in the expansion of the activated lymphocytes directed to the antigen under attack (Grey et al., Sci. Am., 261 38-46, 1989).

Self antigens are also continuously processed and presented as antigen fragments by the MHC glycoproteins to T cells (Jardetsky et al., Nature 353 326-329, 1991). Self recognition thus is intrinsic to the immune system. Under normal circumstances the immune system is tolerant to self antigens and activation of the immune response by these self antigens is avoided.

When tolerance to self antigens is lost, the immune system becomes activated against one or more self antigens, resulting in the activation of autoreactive T cells and the production of autoantibodies. This phenomenon is referred to as autoimmunity. As the immune response in

general is destructive, i.e meant to destroy the invasive foreign antigen, autoimmune responses can cause destruction of the body's own tissue

The contribution of T cells to autoimmune diseases has been established by several studies In mice, experimental autoimmune encephalomyelitis (EAE) is mediated by a highly restricted group of T cells, linked by their specificity for a single epitope of myelin basic protein (MBP) complexed to an MHC class II molecule In the Lewis rat, a species with high susceptibility to various autoimmune diseases, disease has been shown to be mediated by T cells In humans autoimmune diseases are also thought to be associated with the development of auto-aggressive T cells

A destructive autoimmune response has been implicated in various diseases such as rheumatoid arthritis (RA), in which the integrity of articular cartilage is destroyed by a chronic inflammatory process resulting from the presence of large numbers of activated lymphocytes and MHC class II expressing cells The mere presence of cartilage appears necessary for sustaining the local inflammatory response it has been shown that cartilage degradation is associated with the activity of cartilage-responsive autoreactive T cells in RA (Sigall et al , Clin. Exp Rheumat 6 59, 1988, Glant et al , Biochem Soc Trans 18 796, 1990, Burmester et al , Rheumatoid arthritis Smolen, Kalden, Maini (Eds) Springer-Verlag Berlin Heidelberg, 1992) Furthermore, removal of cartilage from RA patients by surgery was shown to reduce the inflammatory process (G.S Panayi et al, Clin Exp Rheumatol 11(suppl 8) S1-S8, 1993) The cartilage proteins are therefore considered to be target autoantigens which are competent of stimulating T cells Activation of these autoreactive T cells leads to development of autoimmune disease

The inflammatory response resulting in the destruction of the cartilage can be treated by several drugs, such as for example steroid drugs However, these drugs are often immunosuppressive drugs that are nonspecific and have toxic side effects The disadvantages of nonspecific immunosuppression makes this a highly unfavourable therapy

The antigen-specific, nontoxic immunosuppression therapy provides a very attractive alternative for the nonspecific immunosuppression This antigen-specific therapy involves the treatment of patients with the target autoantigen or with synthetic T cell-reactive peptides

derived from the autoantigen. These synthetic peptides correspond to T cell epitopes of the autoantigen and can be used to induce specific T cell tolerance both to themselves and to the autoantigen. Although it seems paradoxical to desensitize the immune system with the very same antigen responsible for activating the immune system, the controlled adminstration of the target (auto)antigen can be very effective in desensitization of the immune system.

To effectively use the tolerance therapy to treat the T cell mediated cartilage destruction, there is a great need to identify the responsible autoantigen and to find T cell-reactive peptides which can desensitize patients against the autoantigen that is activating the T cells responsible for the inflammatory process.

It is an object of the invention to provide the autoantigen and T cell reactive peptides derived from said autoantigen which are able to induce specific T cell tolerance to the responsible cartilage antigen in patients suffering from T cell-mediated cartilage destruction. It is another object of the invention to provide a method for detecting autoreactive T cells involved in the destruction of articular cartilage and test kits to be used in said method.

It was surprisingly found that Human Cartilage glycoprotein 39 (herein after referred to as HC gp-39) is a target autoantigen in RA patients which activates specific T cells, thus causing or mediating the inflammatory process. HC gp-39 derived peptides were predominantly recognized by autoreactive T cells from RA patients but rarely by T cells from healthy donors, thus indicating that HC gp-39 is an autoantigen in RA. The arthritogenic nature of HC gp-39 was further substantiated in the Balb/c mouse. A single, subcutaneous injection of said protein in Balb/c mice was able to initiate arthritic signs in the animals. The course of the HC gp-39-induced disease was characterized by relapses occuring periodically in fore paws and/or hind paws and gradually developed from a mild arthritis into a more severe form. Also, a symmetrical distribution of afflicted joints was observed which is, together with the observation of recurrent relapses and nodule formation, reminiscent of disease progression in arthritis, especially RA.

Even more surprisingly it was found that administration of HC gp-39 resulted in immunological tolerance and, more importantly, in delayed and/or suppressed arthritic development.

HC gp-39 is present in serum of both patients and healthy adults, although the serum concentration of the protein is about twice as much in patients as compared to healthy adults. Furthermore, mRNA coding for HC gp-39 can be found in synovial specimens or cartilage obtained from RA patients, whereas cartilage of healthy adults, obtained at surgery, does not contain a significant amount of said mRNA. When articular chondrocytes and synovial cells are cultured, their major secretory product becomes HC gp-39 (Hakala et al., J. Biol. Chem., Vol. 268, 34 25803, 1993). The arthritogenic nature of HC gp-39 was neither described nor suggested in the Hakala et al publication, nor in any other publication.

A further object of the invention is achieved by peptides comprising a subsequence of the autoantigen HC gp-39, characterized in that said peptides comprise one or more of the amino acid sequences FGRSFTLAS (SEQ ID No. 1), FTLASSETG (SEQ ID No. 2), YDDQESVKS (SEQ ID No. 3) and FSKIASNTQ (SEQ ID No 4).

More specifically, a peptide according to the invention comprises one or more of the amino acid sequences PTFGRSFTLASSE (SEQ ID No. 5), RSFTLASSETGVG (SEQ ID No 6), VGYDDQESVKSKV (SEQ ID No. 7) and SQRFSKIASNTQSR (SEQ ID No. 8).

"Subsequence" is understood to be defined as "a part" and should not be mistaken to encompass the entire protein. Preferably the peptides according to the invention have an amino acid sequence of 9-55 amino acid residues. More preferably the peptides according to the invention have an amino acid sequence of 9-35, in particular 9-25 amino acid residues. Much more preferred are peptides having an amino acid sequence of 9-15 amino acid residues. Highly preferred are peptides having an amino acid sequence of 13 or 14 amino acid residues, such as for example peptides having the amino acid sequences given in SEQ ID No. 5-8.

Multimers of the peptide according to the invention, such as for example a dimer or a trimer, in which the monomer sequences optionally can be separated by spacer residues are also within the scope of the invention. Such multimers provide a multitude of the T cell epitopes given in SEQ ID No 's 1-8.

In the peptides according to the invention the amino acid sequences given in SEQ ID No 1-8 can be flanked by flanking regions which may correspond to the native flanking sites of the corresponding amino acids in the amino acid sequence of the HC gp-39 protein or other

proteins in which said amino acid sequences are present. Alternatively, said flanking regions can also be non-native amino acid sequences made up of random amino acid residues. These non-native flanking sites can be used to stabilize the peptides, thus increasing their biological availability. To increase the biological availability of the peptides according to the invention, non-native flanking sites are preferred.

The amino acid sequences given in SEQ ID No.'s 1-4, more specifically the sequences given in SEQ ID No.'s 5-8 resemble MHC class II restricted T cell epitopes which are present on HC gp-39. MHC class II restricted T-cell epitopes on HC gp-39 are displayed by the regions 103-116, 259-271, 263-275 and 326-338 of the amino acid sequence of HC gp-39 (starting from the methionine in the signal sequence, see Hakala et al. 1993). Thus, according to the invention, the peptides can also be understood to encompass fragments of the autoantigen HC gp-39 which comprise one or more of the above identified MHC Class II restricted T-cell epitopes and they are also within the scope of the invention.

The peptides according to the invention are T-cell reactive peptides, which are recognized by and are able to stimulate activated, autoreactive T-cells. These autoreactive T cells are found in the blood of RA patients but rarely in healthy donors.

Thus, according to the invention HC gp-39 protein or the synthetic peptides, said peptides resembling the MHC Class II restricted T-cell epitopes present on the target autoantigen HC gp-39, are very suitable for use in a therapy to induce specific T-cell tolerance to HC gp-39 in patients suffering from T-cell mediated cartilage destruction, such as for example arthritis, more specifically rheumatoid arthritis.

WO 95/01995 and WO 95/02188 describe the diagnostic use of HC gp-39 as a marker for RA, the arthritogenic nature of HC gp-39 is neither disclosed nor suggested. Nowhere do they hint or suggest towards the use of HC gp-39, fragments thereof or T-cell reactive peptides according to the present invention in the antigen or peptide specific therapy to induce T-cell specific tolerance to the HC gp-39 in the cartilage under attack.

The preparation of the peptides according to the invention is effected by means of one of the known organic chemical methods for peptide synthesis HC gp-39 and the petides can also be prepared with the aid of recombinant DNA techniques

The organic chemical methods for peptide synthesis are considered to include the coupling of the required amino acids by means of a condensation reaction, either in homogeneous phase or with the aid of a so-called solid phase

The condensation reaction can be carried out as follows:

a) condensation of a compound (amino acid, peptide) with a free carboxyl group and protected other reactive groups with a compound (amino acid, peptide) with a free amino group and protected other reactive groups, in the presence of a condensation agent;

b) condensation of a compound (amino acid, peptide) with an activated carboxyl group and free or protected other reaction groups with a compound (amino acid, peptide) with a free amino group and free or protected other reactive groups

Activation of the carboxyl group can take place, inter alia, by converting the carboxyl group to an acid halide, azide, anhydride, imidazolide or an activated ester, such as the N-hydroxy-succinimide, N-hydroxy-benzotriazole, p-nitrophenyl ester, N-hydroxy-benzotriazole ester or pentafluorophenol ester.

The most common methods for the above condensation reactions are the carbodiimide method, the azide method, the mixed anhydride method and the method using activated esters, such as described in The Peptides, Analysis, Synthesis, Biology Vol 1-3 (Ed Gross, E. and Meienhofer, J) 1979, 1980, 1981 (Academic Press, Inc)

Preparation of suitable fragments of above-mentioned peptides according to the invention using the "solid phase" is for instance described in J Amer Chem Soc 85 2149 (1963) and Int J Peptide Protein Res 35 161-214 (1990) The coupling of the amino acids of the peptide to be prepared usually starts from the carboxyl end side For this method a solid phase is needed on which there are reactive groups or on which such groups can be introduced This can be, for example, a copolymer of benzene and divinylbenzene with reactive chloromethyl groups, or a polymeric solid phase rendered reactive with hydroxymethyl or amine-function

A particulary suitable solid phase is, for example, the p-alkoxybenzyl alcohol resin (4-hydroxy-methyl-phenoxy-methyl-copolystrene-1% divinylbenzene resin), described by Wang (1974) J. Am. Chem. Soc. 95 1328. After synthesis the peptides can be split from this solid phase under mild conditions.

After synthesis of the desired amino acid sequence, detaching of the peptide from the resin follows, for example, with trifluoroacetic acid, containing scavengers, for example triisopropyl silane, anisole or ethanedithiol,thioanisol.

The reactive groups which may not participate in the condensation reaction are, as stated, effectively protected by groups which can be removed again very easily by hydrolysis with the aid of acid, base or reduction. Thus, a carboxyl group can be effectively protected by, for example, esterification with methanol, ethanol, tertiary butanol, benzyl alcohol or p-nitrobenzyl alcohol and amines linked to solid support.

Groups which can effectively protect an amino group are the ethoxycarbonyl, benzyloxycarbonyl, t-butoxy-carbonyl (t-boc) or p-methoxy-benzyloxycarbonyl group, or an acid group derived from a sulphonic acid, such as the benzene-sulphonyl or p-toluene-sulphonyl group, but other groups can also be used, such as substituted or unsubstituted aryl or aralkyl groups, for example benzyl and triphenylmethyl, or groups such as ortho-nitrophenyl-sulphenyl and 2-benzoyl-1-methyl-vinyl. A particularly suitable α-amino-protective group is, for example, the base-sensitive 9-fluorenyl-methoxycarbonyl (Fmoc) group [Carpino & Han (1970) J. Amer. Chem. Soc. 92 5748].

A more extensive account of possible protecting groups can be found in The Peptides. Analysis, Synthesis, Biology, Vol. 1 - 9 (Eds. Gross, Udenfriend and Meienhofer) 1979 - 1987 (Academic Press, Inc.).

The protective groups can be split off by various conventional methods, depending on the nature of the particular group, for example with the aid of trifluoroacetic acid or by mild reduction, for example with hydrogen and a catalyst, such as palladium, or with HBr in glacial acetic acid.

As already indicated above, HC gp-39 and the peptides according to the invention can likewise be prepared with the aid of recombinant DNA techniques. For this purpose, a nucleic acid sequence which codes for HC gp-39 or a peptide according to the invention or a multimer of said peptide is inserted into an expression vector. Suitable expression vectors are, amongst others, plasmids, cosmids, viruses and YAC's (Yeast Artificial Chromosomes) which comprise the necessary control regions for replication and expression. The expression vector can be brought to expression in a host cell. Suitable host cells are, for instance, bacteria, yeast cells and mammalian cells. Such techniques are well known in the art (Sambrook et al., Molecular Cloning. a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989)

Although it seems paradoxical to desensitize the immune system with the very same antigen responsible for activating the immune system, the controlled administration of HC gp-39 and/or peptides comprising a subsequence of HC gp-39 can be effective in desensitization of the immune system. According to the invention, patients in which the cartilage is under attack of autoresponsive T cells can be treated with a pharmaceutical composition comprising HC gp-39, or one or more peptides according to the invention and a pharmaceutical acceptable carrier in order to make the specific autoreactive T cells of these patients tolerant to the HC gp-39 in the cartilage under attack and other self antigens carrying the identified T cell epitopes having one of the amino acid sequences given in SEQ ID No. 1-8 and to diminish the inflammatory response. Very suitable peptides to be used in a pharmaceutical composition according to the invention are the peptides having an amino acid sequence given in SEQ ID No. 5, 6, 7 and 8.

Also very suitable to be used in a pharmaceutical composition according to the invention are DNA (expression)vectors comprising DNA which encodes for HC gp-39 or one or more of the peptides according to the invention. Upon delivery the DNA (expression)vector can provide by expression a level of the recombinant HC gp-39 protein or peptides according to the invention which is similar to the level which would be achieved by direct administration of a pharmaceutical composition comprising the HC gp-39 protein or peptides.

The autoantigen and peptides according to the invention have the advantage that they have a specific tolerizing effect on the autoreactive T cells thus leaving the other components of

the immune system intact as compared to the nonspecific suppressive effect of the immunosuppressive steroid drugs Treatment with the autoantigen or peptides according to the invention will be safe and no toxic side effects will occur

Tolerance can be attained by administering high or low doses of the autoantigen or peptides according to the invention. The amount of autoantigen or peptide will depend on the route of administration, the time of administration, the age of the patient as well as general health conditions and diet

In general, a dosage of 0.01 to 1000 µg of peptide or protein per kg body weight, preferably 0 5 to 500 µg, more preferably 0 1 to 100 µg of peptide or protein can be used

Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile salin, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water Other carriers may be, for example MHC class II molecules, if desired embedded in liposomes

In addition the pharmaceutical composition according to the invention may comprise one or more adjuvants Suitable adjuvants include, amongst others, aluminium hydroxide, aluminium phosphate, amphigen, tocophenols, monophosphenyl lipid A, muramyl dipeptide and saponins such as Quill A The amount of adjuvant depends on the nature of the adjuvant itself

Furthermore the pharmaceutical composition according to the invention may comprise one or more stabilizers such as, for example, carbohydrates including sorbitol, mannitol, starch, sucrosedextrin and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates

Suitable administration routes are intramuscular injections, subcutaneous injections, intravenous injections or intraperitoneal injections, oral and intranasal administration Oral and intranasal administration are preferred administration routes

Due to its arthritogenic nature, HC gp-39 or the peptides according to the invention can be used to induce clinical arthritis in non-human mammalians. Upon administration of small amounts of HC gp-39 or one or more of the peptides according to the invention, arthritic signs will develop in said mammalians resulting in a disease pattern reminiscent of disease progression in arthritis, especially rheumatoid arthritis When Balb/c mice were injected subcutaneously with HC gp-39 protein, the animals developed arthritic signs The course of the HC gp-39-induced

disease was characterized by relapses occuring periodically in fore paws and/or hind paws and gradually developed from a mild arthritis into a more severe form. Also, a symmetrical distribution of afflicted joints was observed which is, together with the observation of recurrent relapses and nodule formation, reminiscent of disease progression in arthritis, especially RA.

Thus, these afflicted animals provide an adequate animal model to study the mechanism underlying the initiation and progression of arthritic development. Additionally, said afflicted animals can be used to search for new drugs to treat arthritis and to study the effect of these drugs upon the arthritic development. Preferably mice are used as animal model for arthritis, especially rheumatoid athritis.

To induce arthritis in said mammalians, suitable amounts of HC gp-39 or one or more of the peptides according to the invention have to be administered. Suitable amounts are 0.1-1000 µg, preferably 1-100 µg, more preferably 10-50 µg per kg body weight. The amount of HC gp-39 or the peptides will depend on the route of adminstration, time of administration and the type of animal used. Suitable adminstration routes are the same as described before. To induce the effect of arthritis inducton, the HC gp-39 protein or peptides according to the invention may comprise one or more stabilizers or adjuvants as described before.

HC gp-39 or the peptides according to the invention are also very suitable for use in a diagnostic method to detect the presence of activated autoreactive T cells involved in the chronic inflammation of the articular cartilage.

The diagnostic method according to the invention comprises the following steps:

a) isolation of the peripheral blood mononuclear cells (PBMC) from a blood sample of an individual,

b) culture said PBMC under suitable conditions,

c) incubation of said PBMC culture in the presence of the autoantigen or one or more peptides derived thereof according to the invention, and

d) detection of a response of T cells, for example a proliferative response, indicating the presence of activated autoreactive T cells in the individual.

In case of detection of a response by measuring the proliferative response of the autoreactive T cells, the incorporation of a radioisotope such as for example ^{3}H-thymidine is a measure for the proliferation. A response of the autoreactive T cells present in the PBMC can

also be detected by measuring the cytokine release with cytokine-specific ELISA, or the cytotoxicity with ^{51}Chromium release. Another detection method is the measurement of expression of activation markers by FACS analysis, for example of Il-2R. A diagnostic composition comprising one or more of the peptides according to the invention and a suitable detecting agent thus forms part of the invention. Depending on the type of dection, the detection agent can be a radioisotope, an enzyme, or antibodies specific for cell surface or activation markers.

Also within the scope of the invention are test kits which comprise one or more peptides according to the invention. These test kits are suitable for use in a diagnostic method according to the invention.

Thus, the present invention provides for a method to detect whether autoaggressive T cells reactive towards HC gp-39 are present in patients suffering from T-cell mediated cartilage destruction such as for example arthritis, in particular rheumatoid arthritis. If HC gp-39-specific T cells are present, tolerization of these T cells with a pharmaceutical compostion comprising HC gp-39 or peptides according to the present invention or combinations thereof can delay or suppress arthritis development.

The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

LEGENDS TO THE FIGURES

Figure 1. Initiation and progression of arthritis in HC gp-39 tolerized and non-tolerized Balb/c mice. The total arthritic score of the afflicted animals per day following sensitization is given for both fore- and hind paw. The number of afflicted animals per day following sensitization is also given.

EXAMPLE 1

METHODS

Patients

Peripheral blood mononuclear cells (PBMC) from patients who were diagnosed as suffering from RA according to the American Rheumatism Association (ARA) criteria (Arnett et al., Arthritis Rheum 31:315, 1988) were collected. The severity of disease of RA patients ranged from stage I - IV as determined by Rontgenscore

PBMC's from healthy donors carrying the DR4Dw4 (DRB1*0401) or the DR1 specificity were collected as a control. Also PBMC's from two healthy donors who did not carry one of the RA associated DR molecules were collected

MHC typing

Patient and healthy donor PBMC chromosomal DNA extracts, were analysed using the Dynal DR 'low resolution' SSP kit. DR4 subtyping was performed using the Dynal DRB1*04-SSP kit (University Transfusion service, Radboud hospital, Nijmegen, The Netherlands)

Peptides

Peptides according to the invention and a control peptide were synthesized by solid-phase peptide synthesis. In brief, peptides with free amino- and carboxy termini were synthesized on a Milligen 9050 synthesizer, using Fmoc/tBu protected activated esters on PEG-PS resins. After cleaving off and deprotecting, the peptides were purified by preparative HPLC, converted into acetate salts with Dowex Ac-resin or into chloride salts and lyophilized. The peptides were checked with mass spectrometry. The peptides used in this study are listed in table 1. An N-terminal biotinylated Influenza Haemagglutinine derived peptide (SEQ ID No 9), biotin-spacer-IHA(307-319)F, in which the third residue (Y) was replaced by F, (biotin-NH-$(CH_2)_5$-CO-PKFVKQNTLKLAT), was used as marker peptide in the binding studies with DR4Dw4 (DRB1*0401). The non-biotinylated peptide IHA(307-319)F having SEQ ID NO 9 was used as control peptide

Table 1. The amino acid sequence of the synthesized peptides.

peptide	sequence	HPLC purity	SEQ ID
1	PTFGRSFTLASSE	93.2%	No. 5
2	RSFTLASSETGVG	85.6%	No. 6
3	VGYDDQESVKSKV	95.6%	No. 7
4	SQRFSKIASNTQSR	97.6%	No. 8
IHA(307-319)F	PKFVKQNTLKLAT	97.9%	No. 9

The amino acid sequence of peptides 1-4 and the control peptide IHA(307-319)F are given, corresponding to the respective sequence ID's

Cell culture for the production of purified HLA-DR molecules

Two EBV-transformed B-cell lines, BSM (A2, B62, Cw3, DR4Dw4, DQ8, Dpw2) and BM92 (A25, B51, Cw1, DR4Dw14, DQ8), were a gift from the Academic Hospital Leiden, the Netherlands. The cells were cultured in DMEM/HAM's F12 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories), 1% non-essential amino acids (ICI), L-glutamine, 2-ME and antibiotics. Cells were routinely passaged every 2-3 days in a 1:2 ratio. Cells were harvested and thereafter washed three times in PBS (4°C) containing 1 mM PMSF. Cell pellets were stored at -70°C until use.

Affinity purification of HLA-DR molecules

HLA-DR molecules were affinity purified from cell lysates using monoclonal antibody L243 (ATCC HB55), directed against a nonpolymorphic determinant on the DR-complex (Lampson et al., J. Immunol. 125:293, 1980). Protein G sepharose purified L243 was coupled to NHS-Sepharose 4 FF (Pharmacia) according to the manufactures instructions.

HLA-DR expressing cells were thawed and lysed on ice for 30 minutes in PBS, 1% NP-40, 1 mM AEBSF (Calbiochem). The lysate was cleared by centrifugation at 15.000 rpm (Sorvall, SS34 rotor) for 30 minutes. The supernatant was passed through a 0.45 µm filter and added to L243-NHS-sepharose beads. After overnight incubation, the beads were transferred to a column and washed with five volumes PBS, 1% NP-40, 5 volumes PBS, 0.5% NP-40, 15 volumes PBS, 0.5% NP-40, 0.1% SDS, 5 volumes PBS, 0.05% NP-40, 5 volumes PBS, 1% n-

octyl-glucoside (Sigma, St. Louis, USA) and 5 volumes 50 mM diethylamine (Fluka), 150 mM NaCl, 1% n-octyl-glucoside pH=8.0. HLA-DR molecules were eluted with 50 mM diethylamine, 150 mM NaCl, 1% n-octyl-glucoside pH=11. Immediately after collection, the fractions were neutralised with 2M glycine pH=4.8. Collected fractions were analysed on SDS-PAGE under non-reducing conditions followed by silver staining. Fractions containing purified HLA-DR were concentrated by ultrafiltration over a 30 kD cut-off membrane.

HLA-DR peptide binding assay

The peptide binding studies were performed using an improved version of a semi-quantitative binding assay described previously (Joosten et al., Int. Immunol. 6:751, 1994). Purified HLA-DR molecules (0.5-500 nM) were incubated at pH=5.0 with 50 nM biotinylated marker peptide (biotin-spacer-IHA(307-319)F) and a concentration range of competitor peptide (peptides 1-4 and IHA(307-319)F as a control peptide) in a final volume of 25 µl binding buffer (PBS, 1 mM AEBSF, 1 mM N-ethyl maleimide, 8 mM EDTA, 10 µM pepstatin A, 0.01% NaN_3, 0.05% NP-40 and 5% DMSO).

After approximately 45 hours incubation at room temperature, bound and unbound marker peptides were separated either by SDS-PAGE in combination with blotting on a nitrocellulose filter (BioRad) or by vacuum DOT blotting using a nitrocellulose filter (BioRad) and 96 wells Hybry Dot equipment (BRL). Blots were blocked with 0.5% DNA blocking reagent (Boehringer Mannheim, Germany) in 0.1 M maleic acid pH=7.5, 150 mM NaCl. After 1/2 hour, blots were washed in PBS, 0.02% Tween 20 (Sigma, St. Louis, USA) and incubated with Streptavidin-HRPO (Southern Biotechnology) in a 1:40,000 or 1:5,000 dilution respectively. DR-bound, biotinylated marker peptide was detected by enhanced chemoluminescence using a Western Blot ECL kit (Amersham, U.K.) according to the manufactures instructions. Preflashed films (hyperfilm-ECL, Amersham, U.K.) were exposed for 10 minutes. The relative binding affinity of a given peptide was related to competition with the marker peptide. This relative affinity was defined as the peptide concentration at which the signal was reduced to 50% ($^{R}IC_{50}$).

In case of the SDS-PAGE, $^{R}IC50$ values were determined by visual inspection. The density of the DOT Blot-spots was analysed using a computing densitometer (Molecular Dynamics, USA) and Image Quant and Excel software.

Proliferative responses of blood mononuclear cells

In order to identify T cell reactivity to peptide sequences within HC gp-39, the peptides 1-4 were tested for their capacity to induce proliferative responses in PBMC from RA patients versus healthy DR1 or DR4 controls.

PBMC obtained from heparinized venous peripheral blood were isolated by standard centrifugation on a Ficoll-Paque gradient. Cells were cultured in three- or four-fold at a concentration of 1.5×10^5 cells/well in medium supplemented with 10% heat-inactivated autologous plasma, L-glutamine, 2-ME and antibiotics in flatbottomed microtiter plates. Cells were incubated in medium alone or in the presence of PHA (2.5 µg/ml) or in the presence of antigens, including the *Candida albicans* extract (recall antigen) (1 µg/ml, 0.1 µg/ml) or one of the peptides 1-4 in concentrations of 100 µg/ml, 25µg/ml or 10 µg/ml. Cultures were incubated in a total volume of 210 µl for 7 days at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were pulsed during the last 18 hours with 0.25 µCi ^{3}H-thymidine.

Definitions

Patients found to respond to both concentrations of at least one peptide tested were ranked as high responders (HR), patients found to respond to at least one of the concentrations of at least one peptide tested were ranked as responders (R). Consequently, patients which did not respond to any of the peptides tested were ranked as non-responders (NR).

The relative binding affinity of a given peptide was related to competition with the marker peptide. This relative affinity was defined as the peptide concentration at which the signal was reduced to 50% ($^RIC_{50}$).

RESULTS

In order to determine T cell reactivity to peptides 1-4, the PBMC proliferative response in RA patients and healthy donors was analyzed.

Sofar, most HR or R to the HC gp-39-derived peptides carried the DR1, DR4Dw4, DR4Dw14 or the DR10 specificity, all of which are known to be associated with an increased risk for the development of RA. Binding of the peptides 1-4 to DR4Dw4 and DR4Dw14 was demonstrated as shown in Table 2.

Table 2: Binding of the peptides 1-4 to HLA-DR molecules

Peptide	DR4Dw4		DR4Dw14	
	SDS-PAGE	DOT Blot	SDS-PAGE	DOT Blot
1	0.04	0.04	2.5	1.95
2	0.04	0.08	0.2	0.19
3	1	0.23	>=50	>10
4	-	0.08	-	0.4
IHA(307-319)F	0.3	0.55	0.3	0.53

Binding of peptides 1-4 having amino acid sequence given in SEQ ID No. 5-8 respectively to HLA-DR molecules. Peptide binding was determined in a semiquantitative binding assay (as described in Methods of Example 1) at pH=5, using 50 nM IHA(307-319)F as a marker peptide. The values are $^{R}IC_{50}$ values in micromolar (concentration competitor peptide at which the signal is reduced to about 50% of the signal without competitor peptide). All results, except for peptide 4, are from two independent experiments (SDS-PAGE and DOT Blot, respectively).

Most RA patients responded to one or more of the peptides according to the invention (Table 3), whereas responses were rarely found in the healthy donor group (Table 4). 6 RA patient were found not to respond to any of the peptides tested (results not shown).

Disease severity in RA patients was ranked from stage I-IV. The rontgenscore as here indicated is an estimation of the degree of joint destruction. HR and R to the peptides 1-4 were found in all stages of disease.

The peptides according to the invention represent autoreactive T cell epitopes and reactivity to these epitopes was found predominantly in RA patients but rarely in healthy donors. Thus, RA patients have activated, autoreactive T-cells directed at the autoantigen HC gp-39. Clearly the autoantigen HC gp-39 is under attack of T-cells, resulting in inflammation and destruction of the HC gp-39 antigen in the cartilage.

Table 3: Proliferative responses of PBMC from RA patients to peptides 1-4 measured in 10% autologous serum

Patient	Peptide 1			Peptide 2			Peptide 3			Peptide 4			Disease state
	100 µg/l	25 µg/l	10 µg/l	100 µg/l	25 µg/l	10 µg/l	100 µg/l	25 µg/l	10 µg/l	100 µg/l	25 µg/l	10 µg/l	
215-0 (HR)	13	5		19	3		6	3					III-IV
240-0 (HR)	9	5		6	5		6	2					II-III
242-0 (HR)	-	9		-	4		3	9					I
243-0 (R)	2		<1	6		1	1		1				III
247-0 (R)	9		1	2		1	-		1				III
248-0 (R)	2		1	1		1	1		1				III
191-03 (HR)	3		2	<2		1	<2		1	<2		<2	IV
272-0 (R)	<2		1	<1		<1	<1		<1	6		<2	III-IV
273-0 (HR)	14		12	6		7	6		1	29		3	IV
275-0 (HR)	2		4	9		2	1		1	5		<2	III
276-0 (HR)	4		3	1		3	1		<1	<2		1	IV, burnt out
280-0 (HR)	11		6	4		1	1		1	2		<2	IV, burnt out

Proliferative responses of PBMC from RA patients to the peptides 1-4 with the amino acid sequence given in SEQ ID No. 5-8 respectively. Results are presented as stimulation index (SI) values (antigen-specific cp5m (mean of measurements) control cp5m (mean of measurements)) (cp5m counts per 5 minutes. SI values _ 2 were regarded as positive, SI values 2 as negative - standard deviations of the mean of measurements exceeded acceptable values, therefore values are not given. HR high responder (responses at both peptide concentrations tested). R responder (responses at the highest peptide concentration tested). Burnt out almost all cartilage has disappeared.

able 4: Proliferative responses of freshley isolated PBMC from healthy donors to peptides 1-4 measured in 10 % autologous serum.

Donor	Peptide1		Peptide 2		Peptide 3		Peptide 4	
	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml
55-03 (NR)	1	1	1	1	1	<2		
56-03 (NR	· 2	2	<1	1	<1	1	· 2	<1
57-03 (NR)	· 2	1	<2	· 2	1	1		
57-04 (R)	-	10	<1	1	-	1	1	1
68 02 (NR)	1	<2	<1	<2	<2	<2		
68-03 (NR)	<< 1	<2	<1	<1	1	1	<1	1
69-02 (NR)			1	1	1	1		
30-01 (NR)	1	1	1	1	1	1		
35-0 (NR)	<2	1	<2	1	<2	1		
35-01 (NR)	1	<2	<1	<1	<2	<1		
36-01 (NR)	<1	<1	1	1	<1	1		
37-0 (NR)	<1	1	<1	· 2	1	<1		
37-01 (NR)	· · 1	· · 1	1	1	1	1	<1	<1
38-0 (NR)	1	1	<1	1	1	1		
38-01 (NR)	· 1	<1	1	1	1	1	<1	1
41-0 (NR)	1	<2	<1	1	<1	<1		
41-01 (R)	2	<1	<1	<1	1	<1	<1	<1

Proliferative responses of PBMC from healthy donors to peptides 1-4 having the amino acid sequences given in SEQ ID NO 5-8 respectively. Results are presented as stimulation index (SI) values (antigen-specific cp5m (mean of measurements) control cp5m (mean of measurements)). SI values 2 were regarded as negative R responder (respones at the highest peptide concentration tested) - standard deviations of the mean of measurements exceeded acceptable values, therefor values are not given

EXAMPLE 2

METHODS

Purification of HC gp-39 from the MG63 osteosarcoma cell line

MG63 cells (human osteosarcoma ATCC CRL 1427) were cultured in cell factories in DMEM/HAM's F12 serum free medium. HC gp-39 was purified from the culture supernatant by heparin affinty chromatography folllowed by super dex 75 chromatography. Purity was checked by SDS-PAGE. In addition, N-terminal amino acid sequencing confirmed that the purified protein was identical to the protein described by Hakala et al.

Arthritogenicity of HC gp-39 in Balb/c mice

10 or 50 µg of purified HC gp-39 in a 100 µl volume PBS (0.5 M NaCl, 0.01 M sodium phosphate buffer, pH 7.5) mixed 1:1 in incomplete Freunds adjuvant (IFA) was injected subcutaneously in the chest region in 2 x 4 female Balb/c mice (Harlan CPB, Zeist, The Netherlands) whereas 4 controls were injected with PBS (1:1 in IFA). Mice were examined daily for clinical signs of arthritis. Severity of arthritis was assessed by scoring each paw from 0-3 (according to the article by Glant et al). In short, score 0 = no changes, score 1 = erythema and swelling, score 2 = swelling and appearance of deformities, score 3 = immobility due to loss of flexion and extension.

Tolerance induction by intranasal administration of HC gp-39

Twenty eight µg of protein was administered intranasally (2 x 10 µl) to 10 female Balb/c mice (anesthetized lightly with Enflurance) using a PT45 micro conduit and a Hamilton syringe. Antigen was administered on day -15, -10 and -5 prior to arthritis induction. Controls (n = 10) were submitted to the same procedure but recieved the vehicle (PBS) only (Table 5).

Immunological tolerance was evaluated by measuring delayed type hypersensitivity (DTH) responses following sensitization as described above on day 0, using 10 µg of protein. Sensitization on day 0 was followed by an injection of 10 µg HC gp-39 in 50 µg volume in the left hind footpad on day 8 (challenge). DTH reactions were measured as the increase in footpad ([swelling left (mm x 10^{-3}) -swelling right (mm x 10^{-3})]/swelling right (mm x 10^{-3})) x 100%. The

footpad swelling was measured using an inhouse designed μmeter at 0, 24 and 48 hr after challenge

Tolerance to arthritis induction in these same mice was then further monitored until day 31 following sensitization and mice were examined daily for clinical signs. Severity of arthritis was assessed as mentioned above.

Table 5. Tolerization scheme.

DAY	HC gp-39 tolerized	non-tolerized
-15	28 μg HC gp-39 intra nasal	PBS
-10	28 μg HC gp-39 intra nasal	PBS
-5	28 μg HC gp-39 intra nasal	PBS
0	10 μg HC gp-39 subcutaneous	10 μg HC gp-39 subcutaneous
8	10 μg HC gp-39 footpad	10 μg HC gp-39 footpad
9	24 hour DTH	24 hour DTH
10	48 hour DTH	48 hour DTH
0-31	score arthritic signs	score arthritic signs

DTH = delayed type hypersensitivity. PBS = 0.5 M NaCl, 0.01 M sodium phosphate buffer, pH 7.5

RESULTS

Arthritogenicity of HC gp-39

Following one injection of 50 μg HC gp-39 mixed with IFA, all mice gradually developed a severe arthritis (Table 6). Signs of arthritis were observed first at day 15-20 after sensitization, in the fore paws of 3 out of 4 animals. The mouse that did not show any signs in the fore paws developed arthritis in the hind paws by day 34 upon sensitization. The course of HC gp-39-induced disease was characterized by relapses occuring periodically in fore paws or hind paws and gradually develloped from mild arthritis into a more severe arthritis (disease progression was followed for 62 days). Very often (>50%) a symmetrical distribution of afflicted joints was observed, meaning that both fore paws or both hind paws showed arthritic signs at the same time

Arthritis induction was similarly achieved using 10 µg instead of 50 µg HC gp-39 mixed with IFA. Arthritic scores, however, were somewhat lower (data not shown). Three out of 4 mice developed a severe arthritis. One mouse showed only mild signs during the duration of the experiment. All through the length of the experiment control mice showed no signs of arthritis.

In aggregate, both 10 and 50 µg of protein were sufficient to induce a progressive arthritis in Balb/c mice. The chronic nature of arthritis induction by HC gp-39, characterized by recurrent relapses in addition to symmetrical affliction of joints is reminiscent of disease progression in rheumatoid arthritis (RA).

Table 6. Initiation and progression of arthritis in HC gp-39 sensitized Balb c mice.

sensitization (n = 4)	arthritis onset (day)		arthritis signs		
	FP	HP	none	mild	severe
PBS contr.	-	-	4	0	0
10 µg HC gp-39	-, 13, 15, 57	32, 34, 36, 43	0	1	3
50 µg HC gp-39	13, 15, 15, 48	29, 34, 34, 53	0	0	4

FP: fore paw; HP: hind paw; contr.: controls; Arthritis signs: none: score: 0; mild: score per animal does not exceed 2; severe: score per animal is ≥ 4

Immunological tolerance measured in the DTH assay

Control mice injected with HC gp-39 at day 0 showed a strong, antigen-specific DTH response, which suggests that a cellular immune response to HC gp-39 was elicited upon sensitization (Table 7). Intranasal administration of HC gp-39, however, completely abrogated DTH responses upon challenge with the autoantigen, thereby showing that the HC gp-39-specific T-cells were indeed tolerized.

Notably, 4 out of 10 animals of the non-tolerized group developed arthritis in the ankle adjacent to the site of challenge. In contrast, the tolerized group did not develop an arthritis in the joints neighbouring the challenged site, thereby suggesting that immunological tolerance to HC gp-39 results in protection against arthritis development.

Table 7. DTH responses to HC gp-39 following tolerization by nasal administration.

teatment	mean % swelling			arthritis ankle
	0 hr	24 hr	48 hr	
controls (n = 10)	-1.3	31.4	38.6	4/10
tolerized (n = 9)	-0.05	3.7	1.5	0/9

Tolerance to arthritis induction or progression

The HC gp-39 tolerized and the non-tolerized Balb/c mice were then further monitored for initiation and progression of arthritis

In all mice of the control (non-tolerized) group, disease was initiated upon sensitization with HC gp-39 (Table 8) Seven mice gradually developed a severe arthritis whereas three mice showed only mild signs (highest score 2) In contrast, five mice of the HC gp-39-tolerized group were protected against disease development during the course of the experiment Furthermore, two animals of the tolerized group showed only mild signs for brief periods of time. Three animals developed a more severe arthritis with scores of 4

Interestingly, in tolerized animals, arthritis onset was delayed in both hind and fore paws by a minimum of 7-9 days respectively (Figure 1) Although fewer animals were affected in the tolerized group, the arthritic score per animal of the fore paws was comparabe to the arthritic score in the non-tolerized animals The arthritic score per animal of the hind paws however, was somewhat lower in the tolerized animals (Figure 1)

Table 8. Initiation and progression of arthritis in HC gp-39-tolerized and non-tolerized Balb c mice.

animals	arthritis onset (day)	arthritis signs		
		none	mild	severe
controls (n = 10)	13-16	0	3	7
tolerized (n = 10)	23-24	5	2	3

Onset arthritis: first signs appear - highest number of animals affected. Arthritis signs: no signs: score = 0; mild: score per animal does not exceed 2; severe: score per animal is ≥ 4

The experiments above demonstrate the arthritogenic nature of HC gp-39 in Balb/c mice The course of HC gp-39-induced disease was characterized by relapses occuring periodically in fore paws and/or hind paws and gradually developed from a mild arthritis into a more severe form. Also, a symmetrical distribution of afflicted joints was observed which is together with the observation of recurrent relapses, reminiscent of disease progression in rheumatoid arthritis. The fierce arthritogenic nature of HC gp-39 was illustrated by a single, subcutaneous injection of 10 or 50 μg of protein which initiated arthritic signs in all animals That HC gp-39 specific T cells are indeed elicited in response to HC gp-39 sensitization was shown by induction of HC gp-39-specific DTH responses. These data were further confirmed by the demonstration of HC gp-39-specific in vitro proliferative responses in animals immunized in the footpad with HC gp-39 (data not shown) Importantly, non-tolerized animals developed arthritis in the ankle neighbouring the injection site, thereby indeed suggesting an involvement of HC gp-39-specific T cells in arthritis induction

Intranasal administration of peptide antigen has been used to induce antigen-specific immune tolerance The experiments showed that intranasal administration of HC gp-39 leads to immunological non-responsiveness. DTH responses following sensitization were completely abrogated in HC gp-39-tolerized mice whereas control mice showed an antigen-specific swelling These observations indicate that adminstration of HC gp-39 leads to peripheral immune tolerance

In non-tolerized animals DTH responses were accompanied by arthritis in the ankle (adjacent to the challenged site) in four out of ten mice. In contrast, the ankles of HC gp-39-tolerized animals were indeed fully protected, thereby suggesting that autoreactive T-cells have been effectively silenced The notion that tolerization with HC gp-39 protects against disease developement was taken further by the observation that 5 out of 10 animals in the tolerized group were entirely protected throughout the length of the experiment Although the other five animals in the group did eventually develop clinical signs, the onset of arthritis was considerably delayed Hence it can be concluded that HC gp-39-specific T cells are involved in the arthritogenic process and more importantly, that by tolerization of these T cells with a pharmaceutical composition according to the present invention arthritis development can be delayed or suppressed

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: AKZO NOBEL N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM
(G) TELEPHONE: 0412-666376
(H) TELEFAX: 0412-650592
(I) TELEX: 37503 akpha nl

(ii) TITLE OF INVENTION: Novel Peptides derived from autoantigen for use in Immunotherapy of Autoimmune Diseases

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Phe Gly Arg Ser Phe Thr Leu Ala Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Phe Thr Leu Ala Ser Ser Glu Thr Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Tyr Asp Asp Gln Glu Ser Val Lys Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Phe Ser Lys Ile Ala Ser Asn Thr Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Pro Thr Phe Gly Arg Ser Phe Thr Leu Ala Ser Ser Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Arg Ser Phe Thr Leu Ala Ser Ser Glu Thr Gly Val Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Val Gly Tyr Asp Asp Gln Glu Ser Val Lys Ser Lys Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ser Gln Arg Phe Ser Lys Ile Ala Ser Asn Thr Gln Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Pro Lys Phe Val Lys Gln Asn Thr Leu Lys Leu Ala Thr
1 5 10

Claims

1 Peptide comprising a subsequence of HC gp-39, said peptide comprising one or more of the amino acid sequences FGRSFTLAS (SEQ ID No. 1), FTLASSETG (SEQ ID No 2), YDDQESVKS (SEQ ID No 3) and FSKIASNTQ (SEQ ID No 4).

2 Peptide according to claim 1, said peptide comprising one or more of the amino acid sequences PTFGRSFTLASSE (SEQ ID No. 5), RSFTLASSETGVG (SEQ ID No 6), VGYDDQESVKSKV (SEQ ID No 7) and SQRFSKIASNTQSR (SEQ ID No. 8)

3 Peptide according to claim 1 or 2 having the amino acid sequence PTFGRSFTLASSE (SEQ ID No. 5)

4 Peptide according to claim 1 or 2 having the amino acid sequence RSFTLASSETGVG (SEQ ID No 6).

5 Peptide according to claim 1 or 2 having amino acid sequence VGYDDQESVKSKV (SEQ ID No 7)

6 Peptide according to claim 1 or 2 having amino acid sequence SQRFSKIASNTQSR (SEQ ID No 8)

7 The HC gp-39 protein or peptides according to any of the claims 1 to 6 for use as a therapeutical substance

8 Pharmaceutical composition comprising the HC gp-39 protein or one or more of the peptides according to any of the claims 1 to 6, and a pharmaceutical acceptable carrier

9 Use of the HC gp-39 protein or one or more of the peptides according to any of the claims 1 to 6 for the manufacture of a pharmaceutical preparation for the induction of

specific T-cell tolerance to the HC gp-39 autoantigen in patients suffering from T-cell mediated cartilage destruction

10 Use of the HC gp-39 protein or one or more of the peptides according to any of the claims 1 to 6 for the manufacture of a pharmaceutical preparation for the induction of specific T-cell tolerance to the HC gp-39 autoantigen in patients suffering from arthritis, more specifically rheumatoid arthritis

11 HC gp-39 protein or one or more of the peptides according to any of the claims 1-6 for use in a method to induce clinical arthritis in non-human mammalians, preferably mice

12 Peptides according to any of the claims 1-6 for use as a diagnostic substance

13 Diagnostic composition comprising one or more of the peptides according to any of the claims 1-6 and a detection agent.

14 A diagnostic method for the detection of activated autoreactive T cells comprising the following steps

a) isolation of the peripheral blood mononuclear cells (PBMC) from a blood sample of an individual,

b) culture of said PBMC under suitable conditions,

c) incubation of said PBMC culture in the presence of HC gp-39, fragments thereof and/or one or more peptides according to any of the claims 1-6, and

d) detection of a response of T cells, indicating the presence of activated autoreactive T cells in the individual

15 Test kit for the detection of activated autoreactive T cells, said test kit comprising HC gp-39 or one or more of the peptides according to any of the claims 1-6

16 Peptide according to any of the claims 1-15 having an amino acid sequence of 9-55 amino acid residues

FIG, 1A: Arthritis score fore paw non tolerized

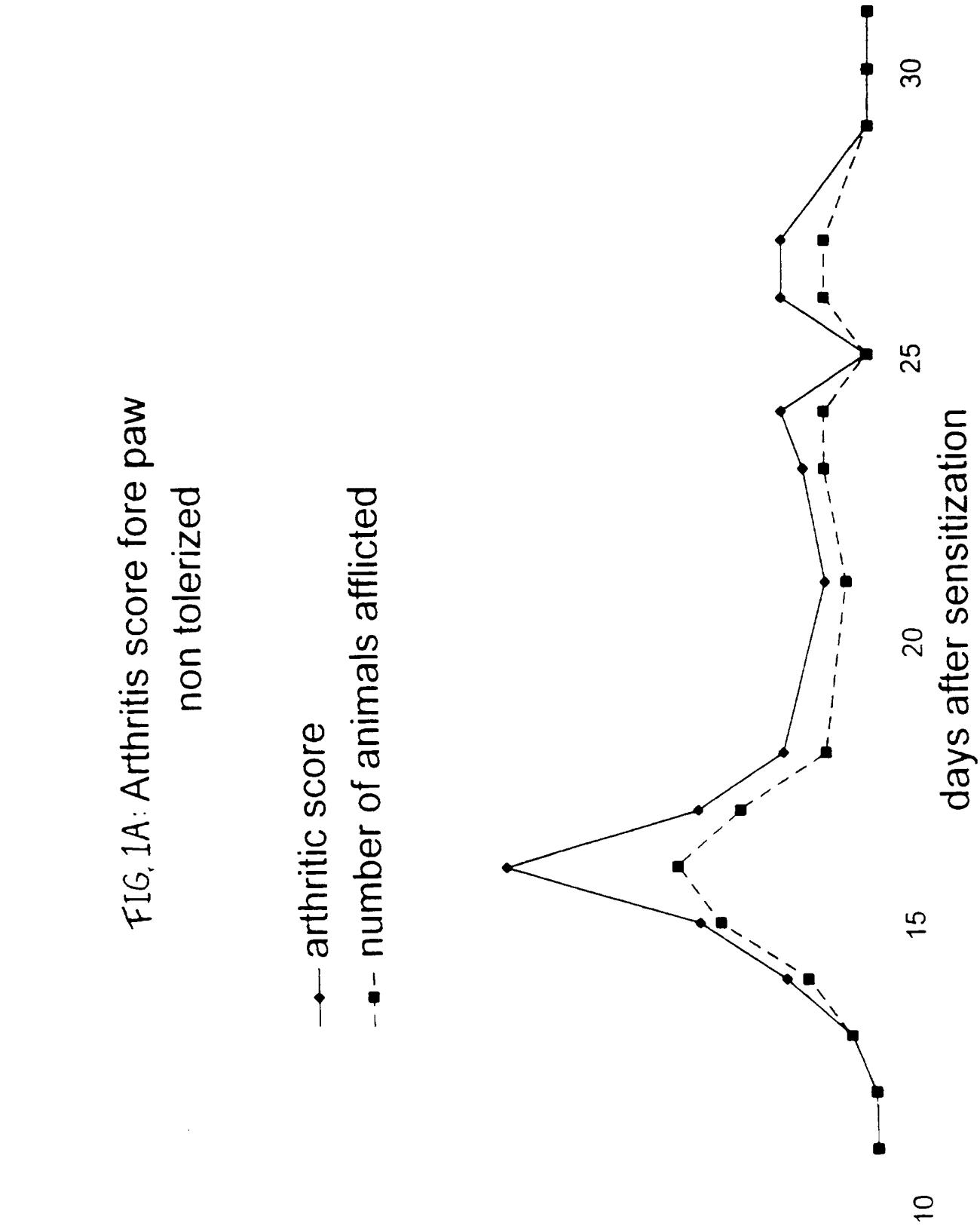


FIG 1B: Arthritis score fore paw tolerized

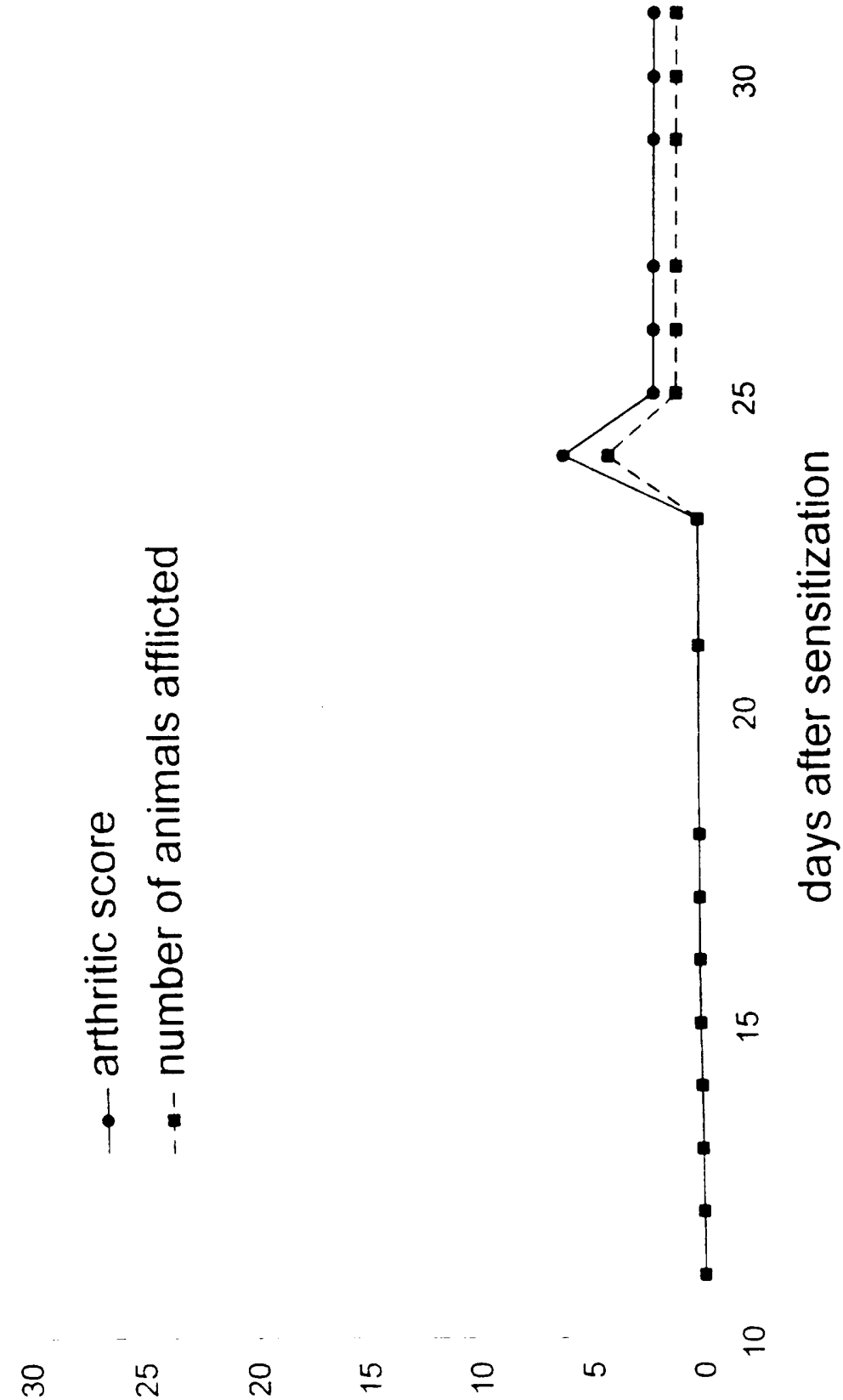


FIG. 1C: Arthritis score hind paw non tolerized

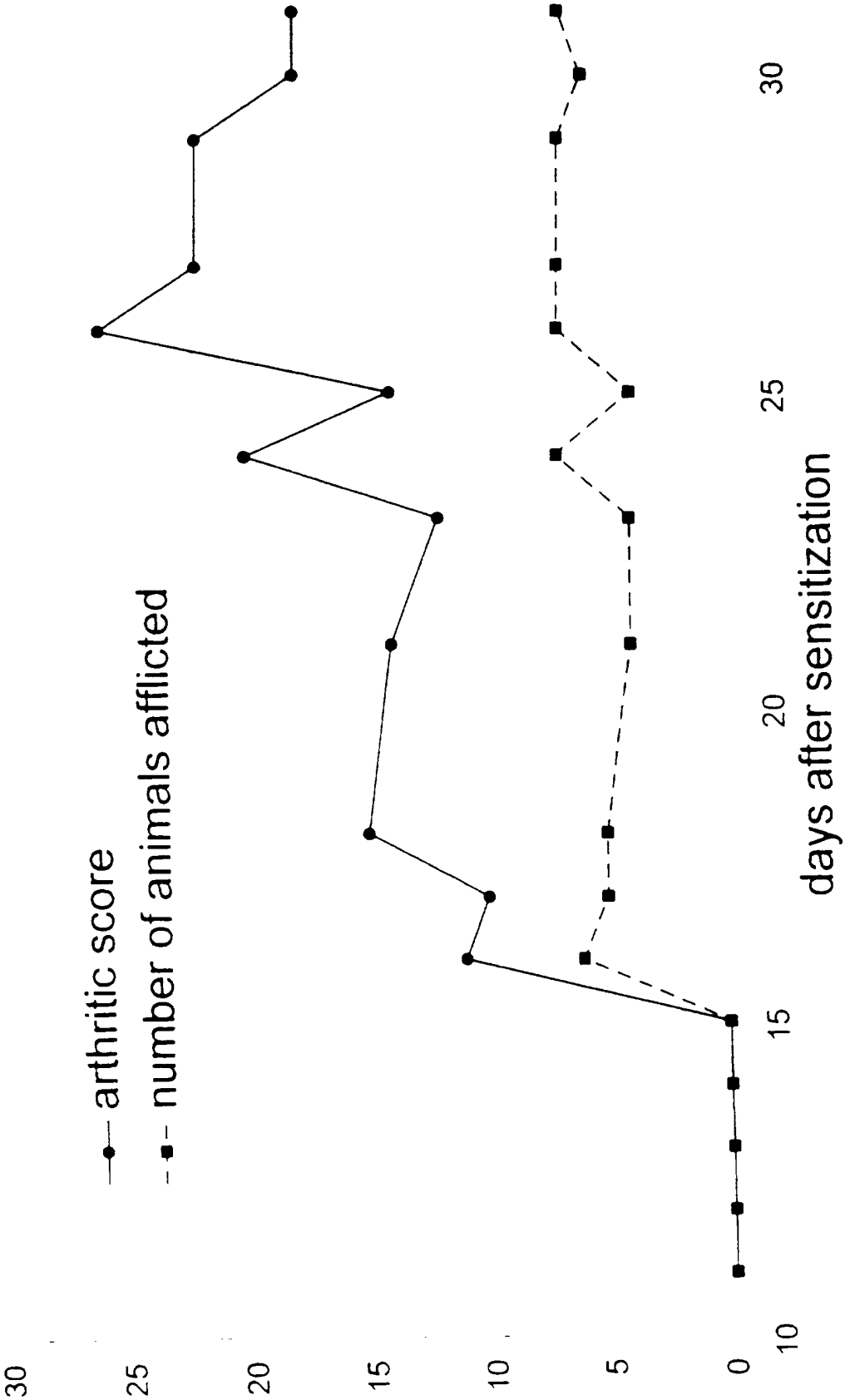
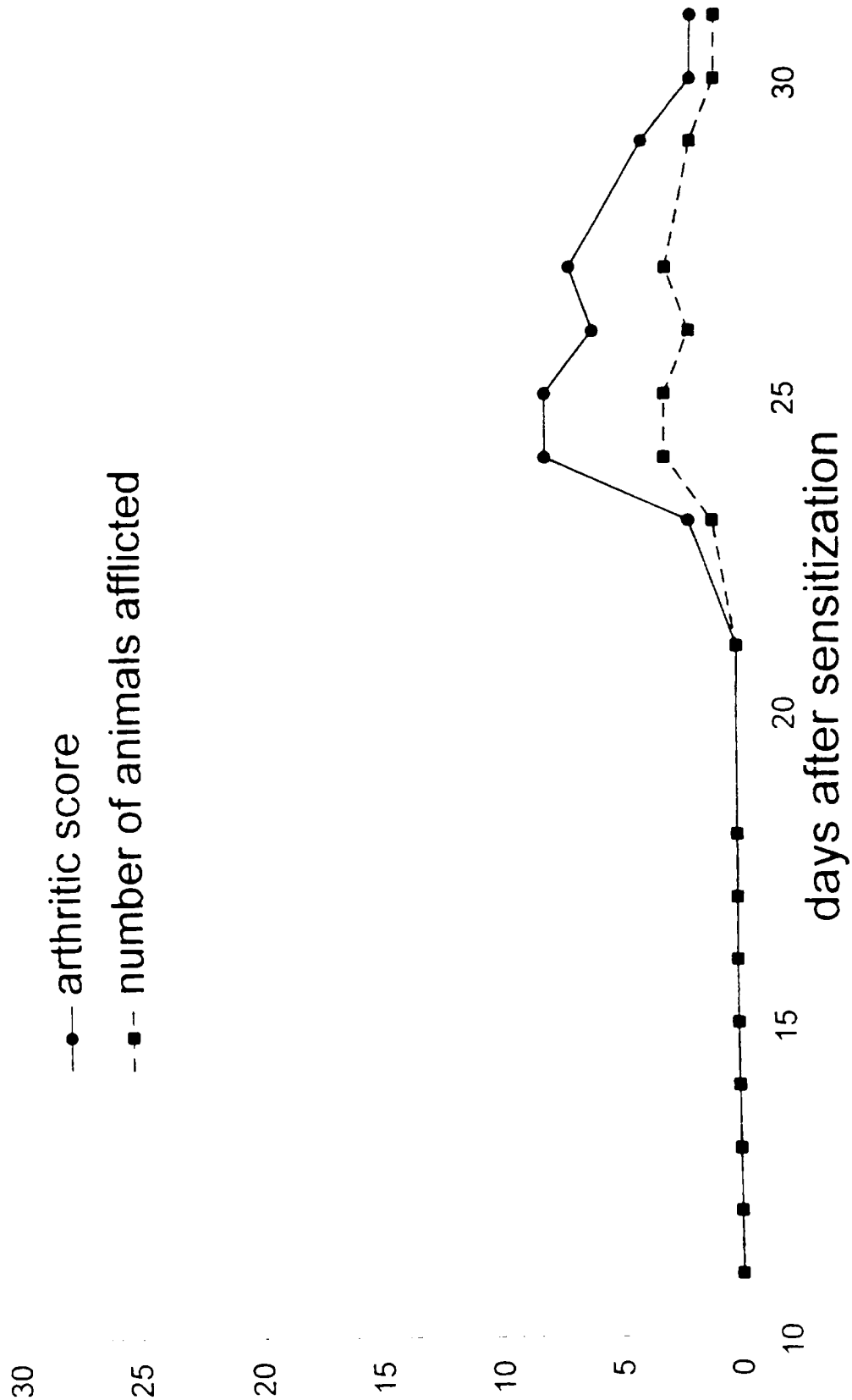


FIG. 1D: Arthritis score hind paw tolerized



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/04201

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A61K38/04 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 34, 5 December 1993 BALTIMORE, MD US, pages 25803-25810, B E HAKALA ET AL. 'Human carilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of chitinase protein family' cited in the application see table 1 --- -/--	1,2,6-10

[X] Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMICAL JOURNAL, vol. 269, no. 1, 1 July 1990 LONDON, THE BIOCHEMICAL SOCIETY, pages 265-268, P NYIRKOS & E E GOLDS 'Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period' see the whole document	1-10
X	DATABASE WPI Week 10 1988 Derwent Publications Ltd., London, GB; AN 88-068419 'New polypeptide and DNA coding-related to protective mechanisms such as immune response..' & JP,A,63 023 898 (DAINIPPON PHARM.) , 16 July 1986 see abstract	1-10, 12-14
X	DATABASE STRAND Genetics Computer Group Sequence, 1 February 1988 T FURUYA ET AL 'cDNA encoding new polypeptide' see abstract & JP,A,88 023 898 (DAINIPPON PHARM.) 1 February 1988	1-10, 12-14
P,X	WO,A,95 01995 (THE REGENTS OF UNIVERSITY OF CALIFORNIA) 19 January 1995 see the whole document	1-16

INTERNATIONAL SEARCH REPORT

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WO-A-9501995	19-01-95	NONE	

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(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL–6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MILTENBURG, Andreas, Martinus, Maria [NL/NL]; Stakenborg 19, NL–5346 VH Oss (NL). BOOTS, Anna, Maria, Helena [NL/NL]; Verlengde Torenstraat 10, NL–5366 AV Megen (NL).

(74) Agent: HERMANS, F., G., M.; P.O. Box 20, NL–5340 BH Oss (NL).

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(54) Title: USE OF HC gp–39 IN IMMUNE DISEASES

(57) Abstract

The present invention relates to the use of HC gp–39 to prevent inflammatory diseases. More specifically, HC gp–39 or fragments thereof can be used to modulate the reactivity of lymphocytes which are reactive to antigens other than HC gp–39 but which are present in the same tissue as where HC gp–39 is being expressed.

*(Referred to in PCT Gazette No. 34/2000, Section II)

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Use of HC gp-39 in immune diseases

The invention pertains to a treatment of (auto)immune disease. More specifically the invention is directed to employing Human Cartilage gp-39 (HC gp-39) as a modulator protein for therapy irrespective of the initial events or causes that led to the initiation of the (auto)immune condition.

The primary functional role of the immune system is to protect the individual against invading pathogens bearing foreign, that is non-self, antigens. In order to fulfill this function in a safe and effective manner, a mechanism is required to discriminate between foreign antigens and autoantigens derived from the individuals own body. Failure of this process of self-non-self discrimination, that is loss of immunological tolerance to self antigens, may lead to immune reactivity to autoantigens resulting in autoimmune disease, involving tissue damage and loss of organ function.

Autoimmune diseases are considered to be a major problem in human health care. Some autoimmune diseases may be the result of an immunological process directed at one antigen or antigenic complex whereas in others the autoimmune reaction may involve many types of antigens that may be present in multiple organs. Several lines of evidence have indicated that the immune system is involved in the pathology of autoimmune diseases. First, the chances of individuals to develop an autoimmune disease are closely linked to their genetic backgrounds: genes encoding major histocompatibility complex (MHC) class II molecules that present (auto)antigens to responding T cells which recognize MHC-peptide complexes show a strong genetic linkage to disease susceptibility. Second, cells of the immune system such as monocyte/macrophages and T cells infiltrate target organs. Third, T cells of patients with autoimmune diseases proliferate in vitro in response to potentially involved autoantigens. Fourth, studies in animal models of autoimmunity have unequivocally demonstrated that cells of the immune system such as monocyte/macrophages and T cells are involved in induction and expression of disease activity.

The immunopathology that may occur in case of an autoimmune disease can be illustrated by a disease as rheumatoid arthritis (RA). RA presents itself as a chronic multisystem disease in which the common clinical manifestation is the persistent inflammatory synovitis accompanied by proliferation of synovial cells, pannus

formation, cartilage degradation and bone erosion, and ultimately joint deformity resulting in loss of function.

Existing therapies for the treatment of autoimmune disorders, such as RA, in which the immune system generates an unwanted and undesirable inflammatory response, are inadequate. Treatment has focused on relief of symptoms of autoimmune disease rather that on its cause. Most drugs used in the treatment of autoimmune diseases, e.g. steroids and non-steroidal anti-inflammatory compounds, are nonspecific and have significant toxic side effects. This is especially problematic since autoimmune diseases are chronic conditions which require the prolonged administration of drugs.

Antigen-specific, nontoxic immunosuppression therapy provides a very attractive alternative for the nonspecific immunosuppression. This antigen-specific therapy involves the treatment of patients with the target autoantigen or with synthetic T cell-reactive peptides derived from the autoantigen. These synthetic peptides correspond to T cell epitopes of the autoantigen and can be used to induce specific T cell tolerance both to themselves and to the autoantigen. Although it seems paradoxical to desensitize the immune system with the very same antigen responsible for activating the immune system, the controlled administration of the target (auto)antigen can be very effective in desensitization of the immune system. Desensitization or immunological tolerance of the immune system is based on the long-observed phenomenon that animals which have been fed or have inhaled an antigen or epitope are less capable of developing a systemic immune response towards said antigen or epitope when said antigen or epitope is introduced via a systemic route.

The human cartilage glycoprotein-39 (HC gp-39) was previously identified as a target autoantigen in RA (Verheijden GFM et al., 1997, Arthritis Rheum., 40, 1115). The autoantigenic nature was demonstrated by selective recognition of DR4 (DRB1*0401) binding peptides by peripheral blood T cells from RA patients.

Using HC gp-39 as the inducing protein, in Balb/c mice a chronic, relapsing model of autoimmune-arthritis was developed. Following sensitization with a single, systemic injection with a low dose (µg range) of HC gp-39 in incomplete Freunds adjuvant (IFA), clinical arthritis developed first in the fore paws. At a later stage, a chronic, second phase of arthritis occurred in the hind paws which waxes and wanes with broad peaks of activity and remained present for at least 250 days. Histological evaluation of hind paws showed marked infiltrates predominantly around the ankle joint. Safranin O staining

revealed depletion of proteoglycans indicating degradation of cartilage. The model was shown to be useful in the investigation of tolerance induction with HC gp-39 as a novel, specific therapy for RA. In this model, nasal application of µg amounts of HC gp-39 several days prior to induction prevented the onset of HC gp-39 induced arthritis. The mechanism behind this is thought to be T-cell mediated since a DTH response to HC gp-39 was completely abrogated. Therapeutic use of HC gp-39 was tested by tolerance induction after the first phase of arthritis had occurred. The data indicate that nasal inhalation of HC gp-39 leads to prevention of a broad peak of arthritis activity in the hind paws. Thus, it has been established that HC gp-39 can, in a highly effective manner, be used to induce antigen-specific immunological tolerance (WO 96/13517).

The main problem in (auto)immune diseases (such as e.g. RA) is that the precise targets or antigens that the immune system is adversely reacting to are largely unknown, implicating that modulating a disease entity in an antigen-specific fashion may not be possible.

It would be an important advantage, however, if an antigen-driven, nontoxic form of immunomodulation therapy could be utilized without knowledge of the antigen(s) that are involved as a target in the (auto)immune response. Such an antigen-driven therapy would involve the generation of antigen-specific modulator cells with the use of an antigen that is expected to be released or produced during the autoimmune process as a result of inflammation or tissue damage. In case of an autoimmune disease, the locally produced autoantigen could then activate or reactivate modulator cells induced with such an antigen.

Evidence indicates that HC gp-39 is being expressed under numerous inflammatory (auto)immune conditions including RA, osteoarthritis (OA), alcohol-induced liver fibrosis, inflammatory bowel disease (IBD) and systemic lupus erythematosus (SLE). In general, HC gp-39 is expected to be expressed in those immune conditions in which monocyte to macrophage maturation occurs (Krause SW et al, 1996, J. Leukoc. Biol. , 60, 540) indicating that potentially in all inflammatory autoimmune diseases HC gp-39 can be found. However, expression of HC gp-39 may not be the direct cause of disease, but the result of localized inflammation.

It has now been found that induction of HC gp-39 reactive modulator cells is beneficial under those conditions in which unwanted immunological activity results in the expression of HC gp-39. Therefore, mucosal administration of HC gp-39 is of

benefit in (auto)immune conditions in general and can be used in the absence of knowledge of the (auto)antigens that are involved in initiation of the inflammatory condition.

According to the present invention it is surprisingly found that HC gp-39 can effectively modulate disease activity irrespective of the antigen(s) that are involved as a target in the (auto)immune response. Thus, it has been found that the mere presence of HC gp-39 at a site of inflammation or in lymph nodes draining such a site, for instance as the result of production during the process of monocyte to macrophage maturation, can result in the activation of modulatory T-cells induced with the use of HC gp-39, and therefore result in downmodulation of disease activity.

It has been demonstrated by Zhang JZ et al (1990, J. Immunol. 145, 2489) that pretreatment of Lewis rats with collagen type II (CII) on days -7, -5 and -2 before induction of disease inhibited arthritis severity in an adjuvant arthritis model (AA model). A pronounced suppression of joint swelling occurred in rats that had been fed with a low amount of CII. This concentration of CII showed very limited effectivity in a treatment protocol (feeding rats three times a week starting at day 17 after arthritis induction) as generally observed in antigen-based immunotherapy of autoimmune diseases.

In contrast to the effcet seen with CII, HC gp-39 does not downmodulate collagen induced arthritis when tested in a scheme designed to prevent the induction of this disease (application on days -15, -10 and -5). Thus, in this situation, HC gp-39 is not effective in downmodulation of arthritis activity when using a pretreatment protocol. Surprisingly, however, HC gp-39 is highly effective in treatment of the autoimmune condition induced with CII when given on days 20, 25 and 30 following arthritis induction. Therefore, when HC gp-39 was given in a therapeutic application schedule that is highly relevant to the clinical situation in which patients present themselves to the doctor with ongoing autoimmune disease, arthritis activity was strongly inhibited. This inhibition of arthritis activity as a result of application of HC gp-39 was much stronger than the effect seen with the antigen used in the induction of disease, collagen type II.

According to the invention HC gp-39 can be used to modulate lymphocytes that are reactive to antigens other than HC gp-39 but are present in the same tissue as HC gp-39. By the induction of antigen-specific T-cell tolerance, autoimmune disorders can be

treated by bystander suppression. More in general, the cells to be modulated are hematopoietic cells. In general, in order to function as a tolerogen the protein must fulfill at least two conditions i.e. it must possess an immune modulating capacity and it must be expressed locally or in lymph nodes draining a local site.

Thus, the present invention provides a method to treat patients suffering from inflammatory autoimmune diseases irrespective of the antigen involved as a target in the immune response, by administration of a pharmaceutical preparation comprising HC gp-39. Such patients may suffer from diseases like Graves' diseases, primary glomerulonephritis, osteoarthritis, juvenile arthritis, Sjögren's syndrome, myasthenia gravis, rheumatoid arthritis, Addison's disease, primary biliary sclerosis, uveitis, systemic lupus erythematosis, inflammatory bowel disease, multiple sclerosis or diabetes. The polypeptide according to the present invention therefore can be used in the preparation of a pharmaceutical to induce tolerance in patients suffering from these diseases. Most preferred patients are treated which suffer from rheumatoid arthritis.

HC gp-39 or fragments thereof can thus be used for the manufacture of a pharmaceutical preparation for modulation of the reactivity of lymphocytes. These lymphocytes may be reactive to antigens other than HC gp-39. They are, however, present in the same tissue as HC gp-39. As a result inflammatory diseases are prevented.

Treatment of autoimmune disorders with HC gp-39 makes use of the fact that bystander suppression is induced to unrelated but co-localized antigens. The regulatory cells secrete in an antigen specific fashion pleiotropic proteins such as cytokines which can downmodulate the immune response.

Optionally a treatment can be combined with the the administration of other medicaments such as DMARDs (Disease Modifying Anti-Rheumatic Drugs e.g. sulfasalazine, anti-malarials (chloroquine, hydroxychloroquine) injectable or oral gold, methotrexate, D-penicillamine, azathioprine, cyclosporine, mycophenolate), NSAIDs (non steroidal anti inflammatory drugs), corticosteroids or other drugs knowns to influence the course of the disease in patients suffering from inflammatory diseases.

It will be clear that also fragments of the tolerogen having the relevant antigenic parts will be sufficient in downregulation of the immune response. Such fragments can be identified by the same assay as described in examples 1 and 2.

The term "fragment" refers to any sequence of amino acids that is part of the polypeptide defined above, having common elements of origin, structure and mechanism of action that are within the scope of the present invention and which are functionally equivalent to the whole antigen.

As used herein, "functional equivalent" means a compound having variations of HC gp-39 or fragments thereof while still maintaining functional i.e. immunological or tolerogenizing characteristics of the sequence of HC gp-39 or the epitope fragments.

The variations that can occur in a sequence may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions that are expected not to essentially alter biological and immunological activities, have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides.

The preparation of the peptides according to the invention is effected by means of one of the known organic chemical methods for peptide synthesis. HC gp-39 and the peptides can also be prepared with the aid of recombinant DNA techniques. For this purpose, a nucleic acid sequence which codes for HC gp-39 or a peptide according to the invention or a multimer of said peptide is inserted into an expression vector. Suitable expression vectors are, amongst others, plasmids, cosmids, viruses and YAC's (Yeast Artificial Chromosomes) which comprise the necessary control regions for replication and expression. The expression vector can be brought to expression in a host cell. Suitable host cells are, for instance, bacteria, yeast cells and mammalian cells. Such techniques are well known in the art (Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989).

The peptides may be stabilised by C- and/or N- terminal modifications, which will decrease exopeptidase catalysed hydrolysis. The modifications may include: C-terminal

acylation, (e.g. acetylation = Ac-peptide), N-terminal amide introduction, (e.g. peptide-NH_2) combinations of acylation and amide introduction (e.g. Ac-peptide-NH_2) and introduction of D-amino acids instead of L-amino acids (Powell et al., J. Pharm. Sci., 81:731-735, 1992).

Other modifications are focussed on the prevention of hydrolysis by endopeptidases. Examples of these modifications are: introduction of D-amino acids instead of L-amino acids, modified amino acids, cyclisation within the peptide, introduction of modified peptide bonds, e.g. reduced peptide bonds $\psi[CH_2NH]$ and e.g. peptoids (N-alkylated glycine derivatives) (Adang et al, Recl. Trav. Chim. Pays-Bas, 113:63-78, 1994 and Simon et al, Proc. Natl. Acad. Sci. USA, 89:9367-9371, 1992).

The controlled administration of HC gp-39 and/or peptides comprising a subsequence of HC gp-39 can be effective in modulation of the immune system. According to the invention, patients in which the tissue is under attack of autoresponsive T cells can be treated with a pharmaceutical composition comprising HC gp-39, or one or more peptides according to the invention and a pharmaceutical acceptable carrier in order to generate T cells in these patients that exert e.g. bystander suppression in such a way that the inflammatory response is diminished.

Very suitable peptides to be used in a pharmaceutical composition according to the invention are the peptides comprising an amino acid sequence given in SEQ ID NO:1 (FGRSFTLAS), SEQ ID NO:2 (FTLASSETG), SEQ ID NO:3 (YDDQESVKS), SEQ ID NO:4 (FSKIASNTQ), SEQ ID NO:5 (PTFGRSFTLASSE, SEQ ID NO:6 (RSFTLASSETGVG), SEQ ID NO:7 (VGYDDQESVKSKV) and SEQ ID NO:8 (SQRFSKIASNTQSR) of WO 96/13517.

Suitable peptides according to the invention are the peptides comprising the SEQ ID NOs 1-8 flanked by sequences up to a total length of 55 amino acids. More preferably the peptides have a length of 25 amino acids. Even more preferably the amino acid sequence of the peptides is identical to the sequence of SEQ ID NO 1-8.

Proteins related to HC gp-39 can similarly be used to develop an autoimmune response. Therefore, it is to be expected that these proteins can also be used for subsequent tolerization. Suitable proteins to be used as an alternative for HC gp-39 in a pharmaceutical composition acccording to the invention are for example pig heparine-

binding 38kDa protein, bovine 39 kDa whey protein, human YKL-39 protein, murine breast regressing 39kDa protein (brp39), human oviduct-specific glycoprotein, murine oviduct-specific glycoprotein, hamster oviduct-specific glycoprotein, bovine oviduct-specific glycoprotein, human chitotriosidase precursor protein and murine secretory protein YM-1 precursor.

Also very suitable to be used in a pharmaceutical composition according to the invention are DNA (expression)vectors comprising DNA which encodes for HC gp-39 or one or more of the peptides or proteins according to the invention. Upon delivery the DNA (expression)vector can provide by expression a level of the recombinant HC gp-39 protein or fragments thereof according to the invention which is similar to the level which would be achieved by direct administration of a pharmaceutical composition comprising the HC gp-39 protein or peptides.

Tolerance can be attained by administering high or low doses of the tolerogen or peptides according to the invention. The amount of tolerogen or peptide will depend on the route of administration, the time of administration, the age of the patient as well as general health conditions and diet.

In general, a dosage of 0.01 to 10000 μg of peptide or protein per kg body weight, preferably 0.05 to 2000 μg, more preferably 0.1 to 100 μg of peptide or protein can be used.

Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water. Other carriers may be, for example MHC class II molecules, if desired embedded in liposomes.

In addition the pharmaceutical composition according to the invention may comprise one or more adjuvants. Suitable adjuvants include, amongst others, aluminium hydroxide, aluminium phosphate, amphigen, tocophenols, monophosphenyl lipid A, muramyl dipeptide and saponins such as Quill A. The amount of adjuvant depends on the nature of the adjuvant itself.

Furthermore the pharmaceutical composition according to the invention may comprise one or more stabilizers such as, for example, carbohydrates including sorbitol, mannitol, starch, sucrosedextrin and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Suitable administration routes are intramuscular injections, subcutaneous injections, intravenous injections or intraperitoneal injections, oral and intranasal administration. Oral and intranasal administration are preferred administration routes. Especially, modulator cells specific for the antigen could be generated by applying the antigen via the mucosae, for instance the nasal mucosae. Mucosal administration of antigens has been shown to induce immunological tolerance to such antigens.

The present invention demonstrates that HC gp-39 can be used to downmodulate autoimmune disease induced with a non-related antigen, thereby supporting the concept that HC gp-39 induced modulator cells can influence autoimmune processes.

The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

Legends to the figures

Figure 1: Effect of nasal application of 30 μg HC gp-39 on onset and progression of collagen type II induced arthritis in DBA/1 mice.

a. Effects on a clinical arthritis score in groups of mice

b. Effects on a clinical arthritis score in individual mice

c. Inhibition of histological alterations as evidenced by infiltrate score

d. Inhibition of histological alterations as evidenced by photograph of knee joint

e. Inhibition of radiological damage as evidenced by X-ray imaging

f. Inhibition of radiological damage as evidenced by photographs of ankle joints

Figure 2: Effect of nasal application of 3, 10 or 30 μg HC gp-39 on onset and progression of collagen type II induced arthritis in DBA/1 mice. Effects on a clinical arthritis score in groups of mice are shown.

Examples

Example 1: Modulation of collagen-induced arthritis by nasal administration of 30 µg of HC gp-39

METHODS

Reagents

Bovine collagen type II was isolated from articular cartilage of knee joints obtained from 1-2 year old calves (Miller EJ, Rhodes RK. In: Colowick SP, Kaplan NO, eds. Methods in Enzymology, Vol 82. New York: Academic Press, 1982:33-65). Collagen was resolved in 0.05M HAc (5 mg/ml) and stored at -70°C. HC gp-39 was isolated from the supernatant of transfected CHO cells. HC gp-39 was purified from the culture supernatant by heparin affinity chromatography followed by superdex 75 chromatography. Purity was checked by SDS-PAGE. The control protein ovalbumin was from Sigma, St. Louis, USA.

Induction and modulation of arthritis

Male DBA/1 mice were obtained from Bomholtgaard. Mice were immunized (day 0) with 100 µg bovine collagen type II in complete Freund's adjuvant (CFA). Mice received an intraperitoneal booster injection with 100 µg bovine collagen type II in saline. On days 20, 25 and 30 mice were treated via the intranasal pathway with either HC gp-39 (n=11; 30 µg/animal/dose), bovine collagen type II (n=11; 100 µg/animal/dose), control protein (n=10; ovalbumin; 100 µg/animal/dose) or with buffer alone (n=10). Nasal tolerance induction was performed under Enflurane anesthesia using a PT45 micro conduit and a Hamilton syringe. A control group (n=10) that was not treated via the nasal pathway was also included in the experiment.

Clinical course of arthritis

Progression of arthritis activity was followed visually over time (days 23, 25, 27, 29, 31 and 34 following arthritis induction) and a score for severity of the disease was given (macroscopic score based on redness and/or swelling in digits and/or paws). At later time points ankylosis was also included in this scoring system. Clinical severity of arthritis was graded on a scale of 0 to 2 for each paw according to the presentation of

redness and/or swelling: score 0: no changes; score 0.5: significant changes; score 1.0: moderate changes; score 1.5: marked changes; score 2.0: severe arthritis accompanied by maximal swelling and redness and later on ankylosis. A further refinement of this score (0.25 increments in the scoring system) has been implemented.

Histopathology

Knee joints were evaluated for the presence of histopathological abnormalities. Joints were removed in toto and fixed for 4 days in 4% formalin. After decalcification in 5% formic acid the specimens were processed for paraffin embedding. Tissue sections (7 μm) were stained with haematoxylin and eosin (HE) in order to detect inflammatory changes or with Safranin O to detect proteoglycan depletion indicative of cartilage breakdown. Histopathological changes were scored according to the following parameters. Infiltration of cells, on a scale of 0 to 3, was assessed based on the amount of inflammatory cells in the synovial cavity and the synovial tissues. Proteoglycan depletion was determined using Safranin O staining, and loss of proteoglycans was scored on a scale of 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. A further score for the progressive loss of articular cartilage, a characteristic parameter of collagen type II induced arthritis, is based on cartilage destruction. This destruction was graded on a scale of 0 to 3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of articular cartilage. Histopathological changes in the knee joints were scored in the patella/femur region on five semi-serial sections of the joint. For the ankle joint, the calcanius region was scored.

Radiology

Radiography (X-ray imaging) was performed on hind paws of individual mice at the end of the experiment (day 34). Radiographs were scored with the use of a stereomicroscope under low magnification. A score of 0 to 5 was given to each paw according to the following guidelines: score 0: no changes; score 1: minor changes; score 2: moderate changes; score 3: marked changes; score 4: severe changes; score 5: complete destruction reflecting the severe arthritis that was externally visible in a number of animals.

RESULTS

Immunomodulatory activity of HC gp-39: clinical effects

DBA/1 mice immunized with collagen type II developed collagen-induced arthritis as planned and expected. Mice treated with the antigen used to induce disease, that is collagen type II, showed a trend towards milder disease activity in the earlier phase of the treatment. Mice treated with HC gp-39 were strongly inhibited in their expression of clinical disease activity (Figure 1A). Inhibition of clinical severity of arthritis was first detectable at day 25 of the experiment and lasted until day 34, at the end of the experiment. Only four of the eleven animals treated with HC gp-39 developed an arthritis that was moderate or severe, seven of the eleven mice were more or less protected (Figure 1B). Mice treated with ovalbumin or buffer showed clinical arthritis that was comparable with the clinical arthritis seen in control mice (Figures 1A and 1B). Thus, the experiment demonstrates that treatment with HC gp-39 can trigger modulatory or regulatory mechanisms that interfere with the induction of arthritis with the use of a non-related antigen, that is collagen type II. Therefore it can be concluded that (auto)immune conditions in which the inciting antigen(s) is not known may benefit from therapeutic application of HC gp-39.

Histological and radiological evaluation

Histological examination of knee joints revealed that inflammatory activity was strongly reduced in HC gp-39 treated animals (Figures 1C and 1D). Proteoglycan depletion, as evidenced by a reduction in staining with Safranin O, was strongly inhibited in HC gp-39 treated animals, as was the breakdown of articular cartilage (Figures 1C and 1D). Examination of radiographs revealed inhibition of cartilage breakdown and bone erosion in those animals that were succesfully treated with HC gp-39 (Figures 1E and 1F). It is concluded that the therapeutic application of HC gp-39 not only modifies the clinical pattern of disease expression but also prevents ongoing histologically and radiographically detectable alterations that reflect inflammatory and erosive processes that lead to joint destruction and loss of function. Thus, treatment with HC gp-39 actually alters the course of the disease.

Example 2: Modulation of collagen-induced arthritis by nasal administration of 3, 10 or 30 μg of HC gp-39

METHODS

Reagents

Bovine collagen type II was isolated from articular cartilage of knee joints obtained from 1-2 year old calves (Miller EJ, Rhodes RK. In: Colowick SP, Kaplan NO, eds. Methods in Enzymology, Vol 82. New York: Academic Press, 1982:33-65). Collagen was resolved in 0.05M HAc (5 mg/ml) and stored at -70°C. HC gp-39 was isolated from the supernatant of transfected CHO cells. HC gp-39 was purified from the culture supernatant by heparin affinity chromatography followed by superdex 75 chromatography. Purity was checked by SDS-PAGE. The control protein ovalbumin was from Sigma, St. Louis, USA.

Induction and modulation of arthritis

Male DBA/1 mice were obtained from Bomholtgaard. Mice were immunized (day 0) with 100 μg bovine collagen type II in complete Freund's adjuvant (CFA). Mice received an intraperitoneal booster injection with 100 μg bovine collagen type II in saline. On days 20, 25 and 30 mice were treated via the intranasal pathway with either 3 μg/animal/dose, 10 μg/animal/dose or 30 μg/animal/dose of HC gp-39 (n=10 per group), bovine collagen type II (n=10; 100 μg/animal/dose) or control protein (n=10; ovalbumin; 100 μg/animal/dose). Nasal tolerance induction was performed under Enflurane anesthesia using a PT45 micro conduit and a Hamilton syringe.

Clinical course of arthritis

Progression of arthritis activity was followed visually over time (days 23, 25, 26, 28 and 30 following arthritis induction) and a score for severity of the disease was given (macroscopic score based on redness and/or swelling in digits and/or paws). At later time points ankylosis was also included in this scoring system. Clinical severity of arthritis was graded on a scale of 0 to 2 for each paw according to the presentation of redness and/or swelling: score 0: no changes; score 0.5: significant changes; score 1.0: moderate changes; score 1.5: marked changes; score 2.0: severe arthritis accompanied by maximal swelling and redness and later on ankylosis. A further refinement of this score (0.25 increments in the scoring system) has been implemented.

RESULTS

Immunomodulatory activity of various doses of HC gp-39: clinical effects

DBA/1 mice immunized with collagen type II developed collagen-induced arthritis as planned and expected. Mice treated with the antigen used to induce disease, that is collagen type II, again showed a trend towards milder disease activity in the earlier phase of the treatment. Importantly, mice treated with 30 μg of HC gp-39 were strongly inhibited in their expression of clinical disease activity (Figure 2). Mice that had been treated with 3 or 10 μg of HC gp-39 also showed inhibition in their expression of clinical disease, although to a lesser extent. Inhibition of clinical severity of arthritis is further detailed by data of hind paws of individual mice experiencing arthritis on evaluation days 23, 25 and 30, again demonstrating clinical effectivity of treatment with HC gp-39, most effectively at the 30 μg/animal dose. Therefore, the experiment demonstrates that treatment with HC gp-39, especially with 30 μg/animal/dose, can trigger modulatory or regulatory mechanisms that interfere with the induction of arthritis with the use of a non-related antigen, that is collagen type II. Thus, it can be concluded that (auto)immune conditions in which the inciting antigen(s) is not known may benefit from therapeutic application of HC gp-39.

Example 3: HC gp-39 responses in RA patients

METHODS

Patients and reagents

Patients were diagnosed as suffering from RA according to the American Rheumatism Association (ARA) criteria (Arnett et al., Arthritis Rheum 31:315, 1988). Severity of disease of these patients was ranged from stage 0 - IV as determined by X-ray score. HC gp-39 was purified from culture supernatant by affinity chromatography followed by superdex 75 chromatography. Purity was checked on SDS-PAGE. Candida albicans was obtained from Hal allergenen lab.

Proliferation assay

Peripheral blood mononuclear cells (PBMC) were collected from heparinized venous peripheral blood by standard centrifugation on a Ficoll-Paque gradient. The assay used to determine proliferative responses to HC gp-39 is a modification of an

assay that had been described in the literature (Salvat et al., J Immunol 153:5321, 1994). In brief, PBMC obtained as described above were suspended in wells of a 24 well plate in a concentration of 5 x 10^5 cells per ml. Cells were incubated in medium alone or in the presence of antigen (HC gp-39 was tested in a dose range of 2, 10, 25 and 50 μg/ml; Candida albicans, a control antigen, was tested at 1 and 10 μg/ml). Spontaneous proliferation or background proliferation was assayed in three different background wells. Cultures were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2. Cells were then suspended and 200 or 150 μl volumes of medium were distributed in 4 or 5 fold in wells of a 96-well round-bottomed plate. Cultures were pulsed with 0.5 μCi (1.85 x 10^4 Bq) [^{3}H]thymidine ([^{3}H]TdR) for the last 18 hr of culture. Cells were harvested on glassfibre filters and [^{3}H]TdR incorporation was measured by gasscintillation. Note that counting by gasscintillation is fivefold less efficient compared to liquid scintillation. Filters were measured for 5 min (Packard Matrix 96 β-counter; Meriden, CT).

RESULTS

Responses of RA patients to HC gp-39

It was tested whether PBMC obtained from patients with RA (n=10) proliferated to HC gp-39. In order to establish the quality of the PBMC preparations under study, in a number of cases Candida albicans was used as a positive control antigen. Six out of ten patients could be classified as being a HC gp-39 responder (SI > 10); four out of ten patients did not respond to HC gp-39 (SI < 10) (Table 1). Both responders (R) and non-responders (NR) were found in various stages of the disease, either having active or non-active disease at the time of blood sampling. Thus, RA patients can be classified into individuals responding to HC gp-39 and individuals not responding to HC gp-39 with the use of a routine proliferation assay.

Table 1: HC gp-39 reactivity in RA patients

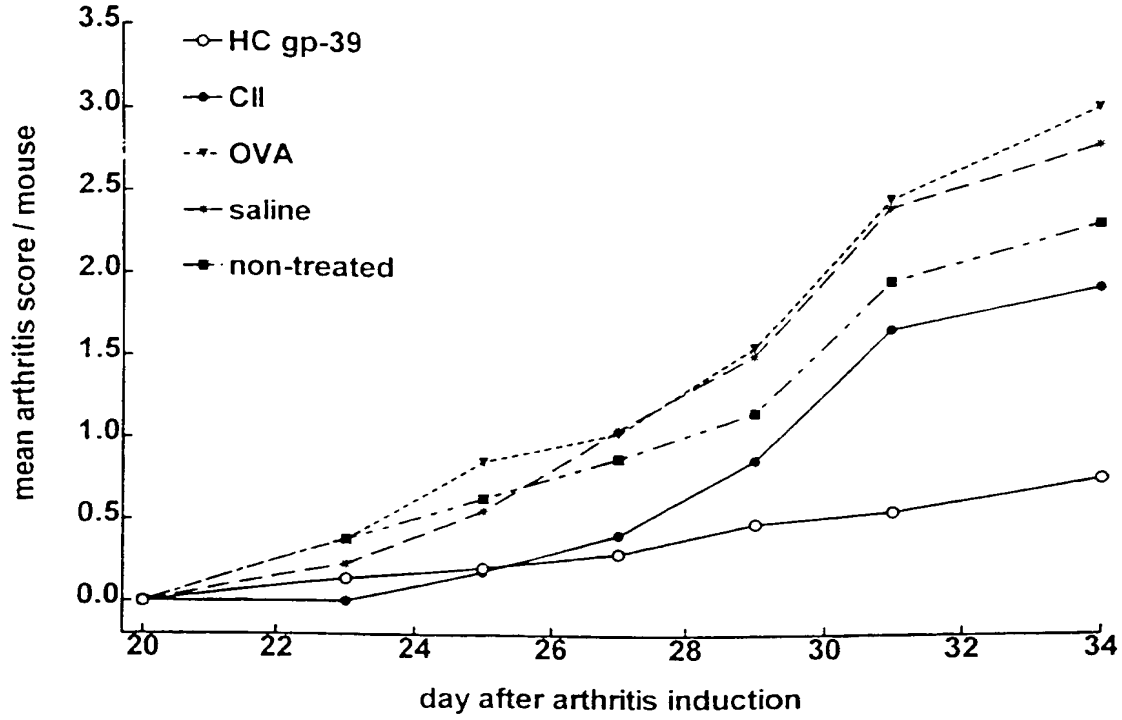
Patient ID	disease stage	BG	HC gp-39 (µg/ml)				*Candida* (µg/ml)		
			(2)	(10)	(25)	(50)	(1)	(10)	R/NR
246-0.2	Iiact	166	2.7	**36**	**61**	**64**	Nd	nd	R
308-0.1	IIIact	200	1.3	**58**	**27**	**12**	Nd	nd	R
403-3	IIIact	193	1.2	**95**	**55**	**59**	Nd	nd	R
406-0.1	Iiact	339	2.4	1.4	2.3	nd	**147**	**126**	NR
407-0	II	120	**20**	**112**	**139**	**100**	Nd	nd	R
421-0	0act	440	2.6	3.5	**46**	**69**	Nd	nd	R
438-0.2	IV	212	0.8	1	0.8	0.-	**20**	**160**	NR
464-0	0act	144	0.9	**167**	**228**	**184**	**89**	**21**	R
470-0.1	0act	113	1.4	1.1	2.5	nd	**368**	**350**	NR
474-0	IIIact	341	4.5	4.2	2.2	nd	**70**	**55**	NR

Disease stage is defined by Steinbrocker criteria. act = active disease if 1 or more joints are inflamed. BG is background counts per 5 minutes. Responses to HC gp-39 are given as SI values. SI values > 5 are considered positive and are indicated in bold. R = responder. NR = non responder. As a control, the response to Candida albicans was determined in order to establish the quality of the PBMC used. nd = not determined

Claims

1. Use of HC gp-39 or fragments thereof for the manufacture of a pharmaceutical preparation for modulation of the reactivity of lymphocytes, said lymphocytes being reactive to antigens other than HC gp-39, said antigens being present in the same tissue as HC gp-39 so as to prevent inflammatory diseases.
2. Use of fragments according to claim 1 characterzized in that the fragments are selected from the group comprising an amino acid sequence given in SEQ ID NO:1 (FGRSFTLAS), SEQ ID NO:2 (FTLASSETG), SEQ ID NO:3 (YDDQESVKS), SEQ ID NO:4 (FSKIASNTQ), SEQ ID NO:5 (PTFGRSFTLASSE, SEQ ID NO:6 (RSFTLASSETGVG), SEQ ID NO:7 (VGYDDQESVKSKV) or SEQ ID NO:8 (SQRFSKIASNTQSR).
3. Use of HC gp-39 or fragments thereof according to claims 1 or 2 for the manufacture of a pharmaceutical preparation for modulation of the reactivity of lymphocytes, said lymphocytes being reactive to antigens other than HC gp-39, said antigens being present in the same tissue as HC gp-39 so as to prevent rheumatoid arthritis.

Figure 1A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OVA
saline
non-treated
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24
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32
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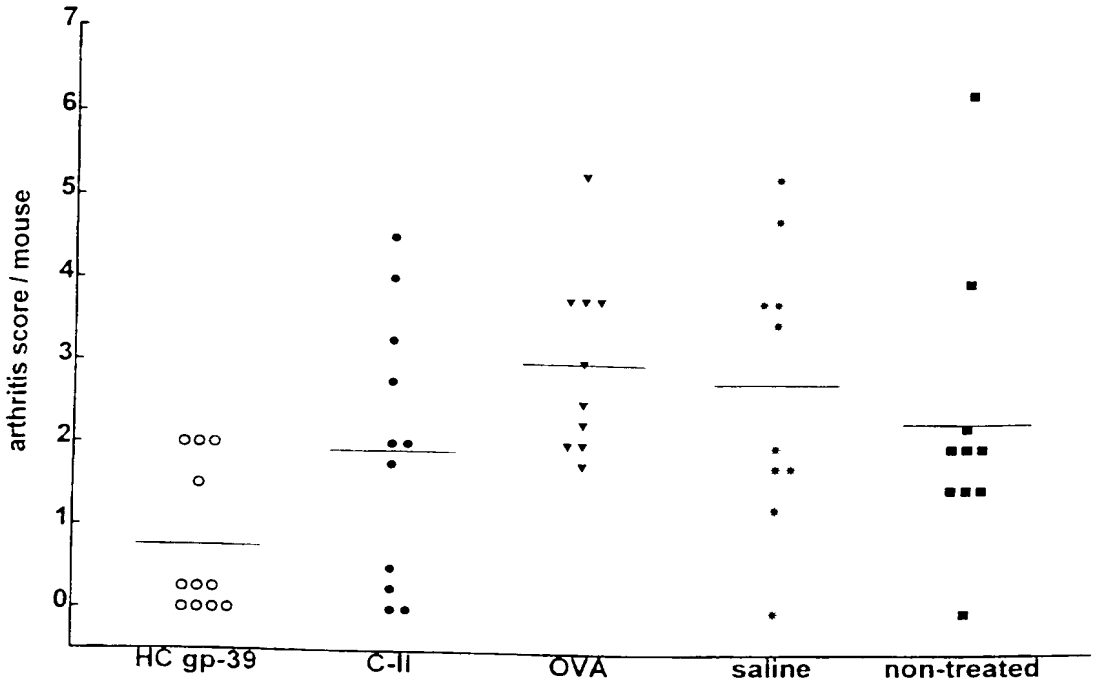
day after arthritis induction

Figure 1B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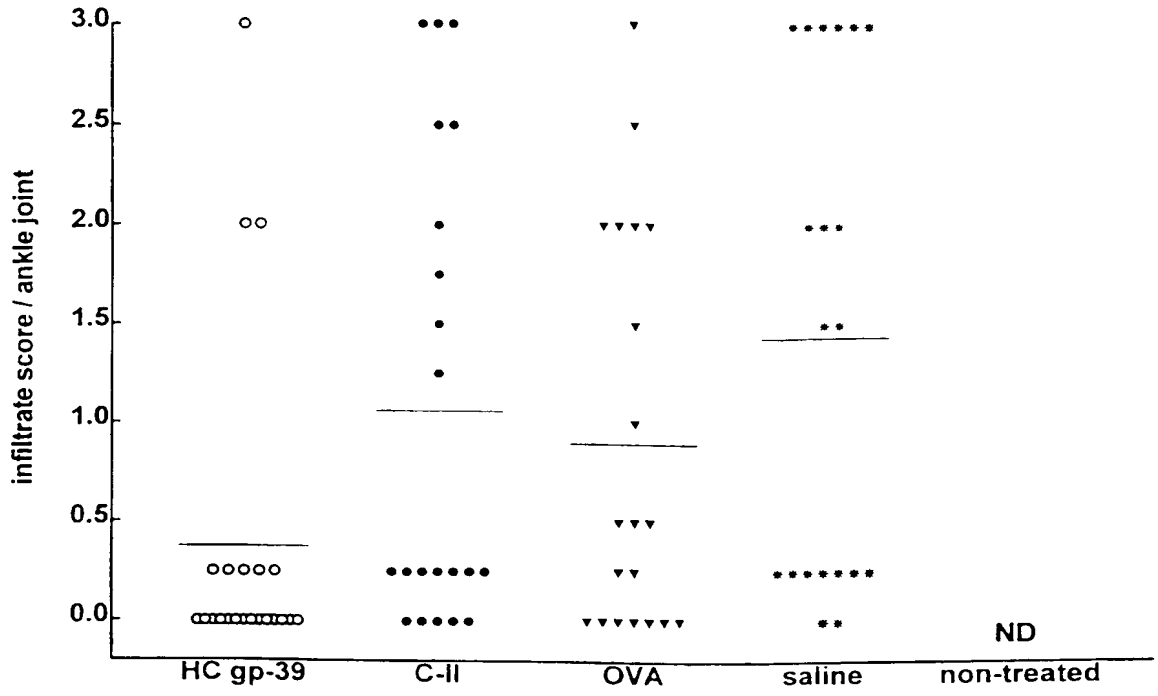
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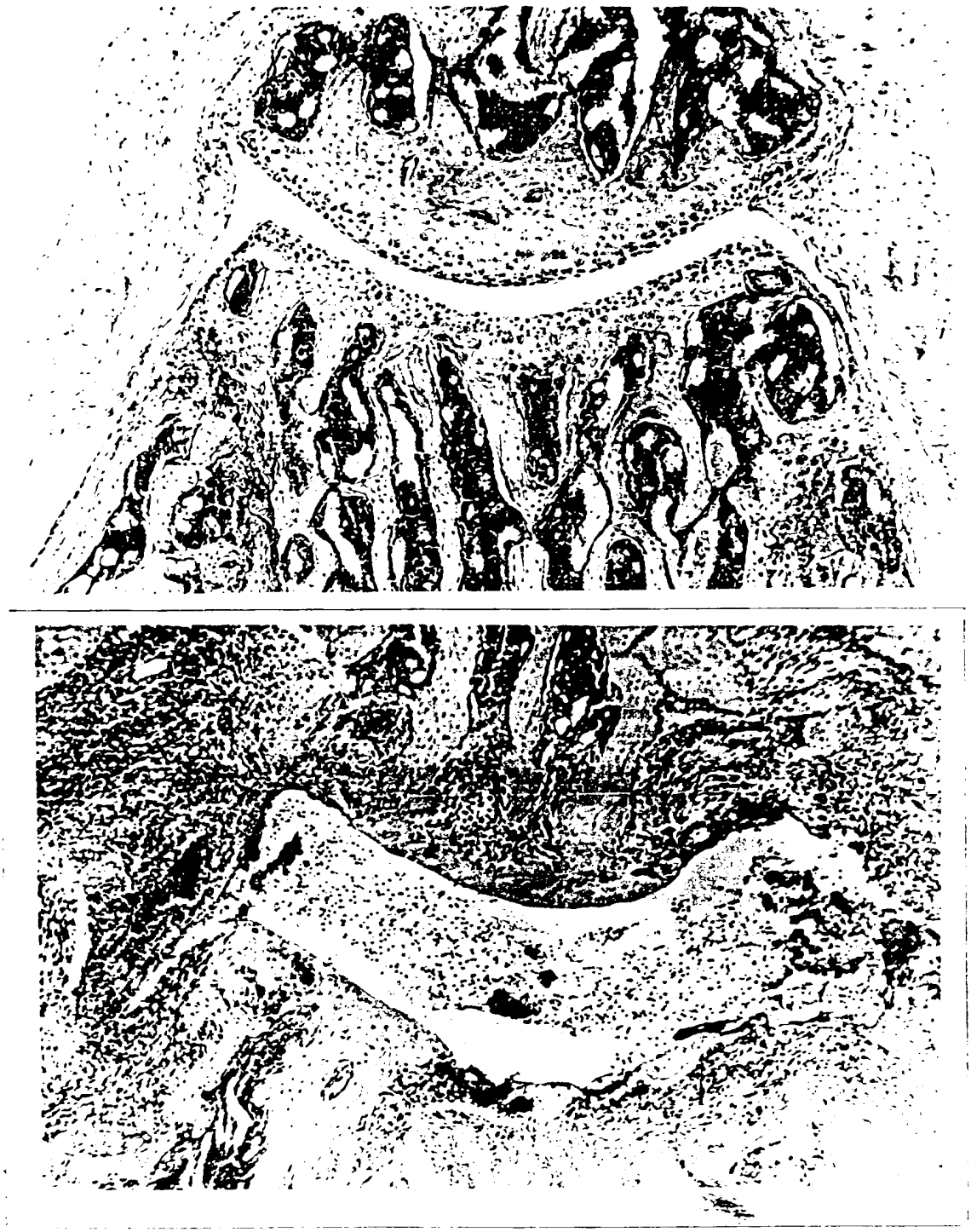
ND

Figure 1D



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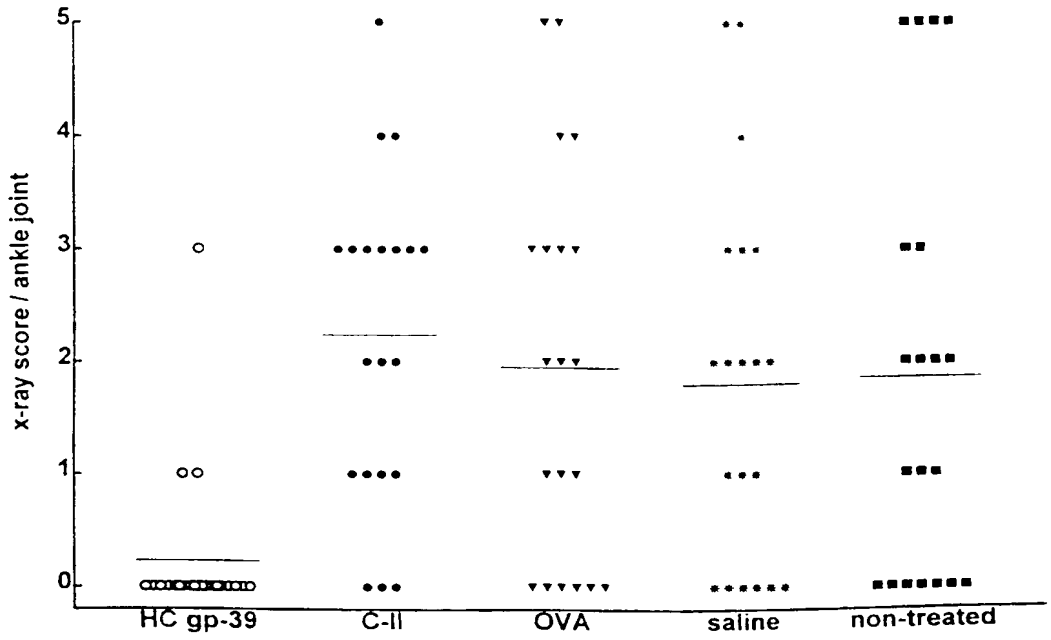
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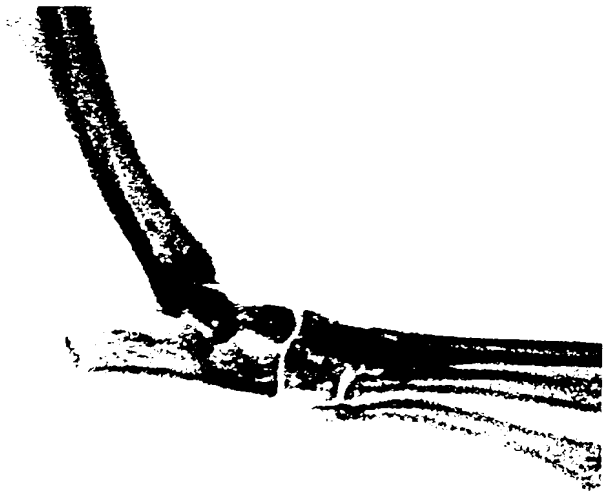


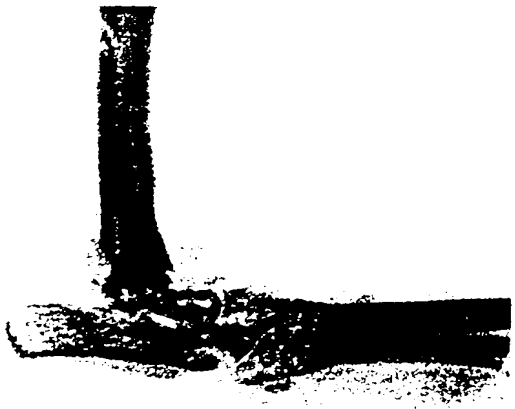
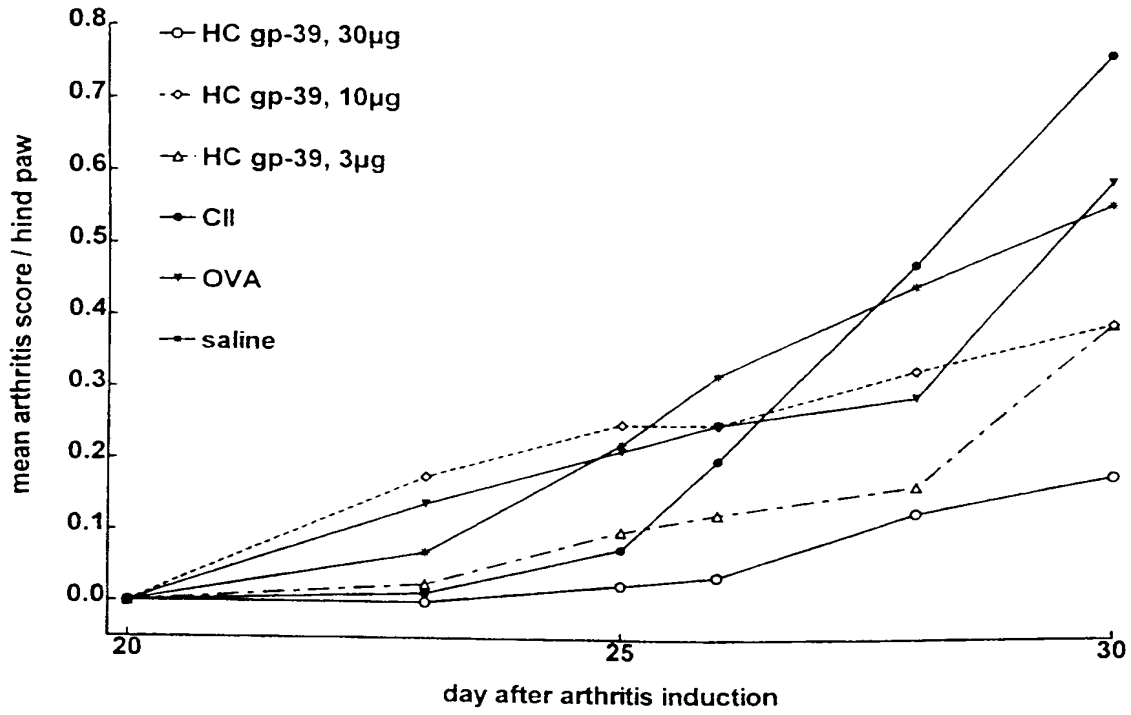


Figure 2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mean arthritis score / hind paw
HC gp-39, 30µg
HC gp-39, 10µg
HC gp-39, 3µg
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OVA
saline
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day after arthritis induction

SEQUENCE LISTING

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<120> Use of HC gp-39 in immune diseases

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 99/05331

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 805 206 A (SMITHKLINE BEECHAM CORPORATION) 5 November 1997 (1997-11-05) the whole document	1,3
X	VERHEIJDEN G F ET AL: "Human cartilage glycoprotein -39 as a candidate autoantigen in rheumatoid arthritis." ARTHRITIS AND RHEUMATISM, (1997 JUN) 40 (6) 1115-25, XP002089334 cited in the application the whole document	1,3
A	EP 0 823 478 A (SMITHKLINE BEECHAM CORPORATION) 11 February 1998 (1998-02-11) the whole document	1
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	04/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/05331

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 96 13517 A (AKZO NOBEL) 9 May 1996 (1996-05-09) cited in the application the whole document	1-3
X	WO 97 40149 A (AKZO NOBEL) 30 October 1997 (1997-10-30) page 4 -page 6	1,3
P,X	COENEN DE ROO C J J ET AL: "Human cartilage GP-39 induces and modifies a chronic relapsing arthritis in mice." 62ND NATIONAL SCIENTIFIC MEETING OF THE AMERICAN COLLEGE OF RHEUMATOLOGY AND THE 33RD NATIONAL SCIENTIFIC MEETING OF THE ASSOCIATION OF RHEUMATOLOGY HEALTH PROFESSIONALS;SAN DIEGO, CALIFORNIA, USA; NOVEMBER 8-12, 1998, vol. 41, no. 9 SUPPL., 1998, page S214 XP002133815 Arthritis & Rheumatism Sept., 1998 ISSN: 0004-3591 the whole document	1-3

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PCT/EP 99/05331

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